

MICROBIAL DEGRADATION OF
CHLORINATED HYDROCARBON INSECTICIDES

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Darrell Jay Grimes

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Darrell Jay Grimes

Approved by Committee:

Genell E. Fair
Chairman
Paul A. Mestrich
Wayne E. Margenroth

Earle I. Canfield
Dean of the Graduate Division

1622-19

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CHAPTER I

INTRODUCTION

Statement of the Problem. The attempt to control arthropods which parasitize man and his domestic animals and which destroy crops and fibers has led synthetic chemists to create a wide variety of insecticides, each with a unique chemical structure and specific use. Use of these compounds gained immediate acceptance upon their development, and today, insecticides are used quite extensively. In 1964, Frear reported that 38,924,345 acres were treated for insect and disease control with Iowa alone accounting for 2,512,058 acres. Treatment of livestock for insect control represented the other major use of insecticides in 1964, with 81,612,955 head being treated in the United States and 10,155,579 head treated in Iowa. These figures include only agricultural acreage and livestock and thus exclude such uses as application to cities for insect control, control of household and lawn pests, and other similiar applications.¹

Fortunately, the bulk of this material is degraded by natural processes shortly after being released into

¹D. E. H. Frear, Pesticide Handbook-Entoma (State College, Pennsylvania: College Science Publishers, 1967), p. 26.

the environment. Unfortunately, however, while the use of insecticides expedites arthropod control, such use many times results in severe and persistent pollution of the environment. In the early 1950's, it became evident that many insecticides were resisting, or were recalcitrant towards, degradation and residues were accumulating in certain areas.¹ These chemicals are now demonstrable in varying concentrations in all phases of the environment from living organisms to the soil and water supplies.

The insecticides classified as the chlorinated hydrocarbons are perhaps the greatest threat to pollution. They remain detectable in the soil in appreciable amounts for long periods of time. One such chlorinated hydrocarbon is chlordane (1,2,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan²). Chlordane is a common household and agricultural insecticide used mainly for the control of locusts, grasshoppers, soil insects, ticks, cockroaches, and flies. Chlordane belongs to the aldrin-toxaphene group

¹D. Chisholm, et al., "DDT Residues in Soil," Journal of Economic Entomology, XLIII (December, 1950), 941; W. E. Fleming and W. W. Maines, "Persistence of Chlordane in Soils of the Area Infested by the Japanese Beetle," Journal of Economic Entomology, XLVII (February, 1954), 165.

²Name conforms to International Union of Physical and Applied Chemistry nomenclature.

of insecticides which includes aldrin, chlordane, dieldrin, endrin, heptachlor, and toxaphene. Frear reported that this group of compounds accounted for 118,832,000 pounds of the 503,861,000 pounds of insecticides, fumigants, and rodenticides produced in the United States in 1965. The figure given for the aldrin-toxaphene group reflected an increase of 13,526,000 pounds over the amount produced in 1964.¹ Chlordane has been reported by many workers to be recalcitrant towards degradation under various conditions. It has also been shown to be toxic to many organisms with LD₅₀'s reported for various invertebrates and vertebrates. Thus, with the knowledge that chlordane resists biodegradation, is toxic to many animals, and that use of it and related compounds seems to be increasing, it is desirable to determine whether or not this insecticide is biodegradable.

Objectives. The objectives of this study are as follows:

1. To determine whether or not chlordane is biodegradable under laboratory conditions, by employing enrichment culture techniques

¹D. E. H. Frear, Pesticide Handbook-Entoma (State College, Pennsylvania: College Science Publishers, 1967), p. 29.

on various soil samples and by analyzing soil samples for increased respiratory activity in the presence of chlordane.

2. To characterize any organism(s) found to degrade chlordane.
3. To elucidate the optimum conditions such as pH, temperature, and insecticide concentration for degradation to occur.

CHAPTER II

HISTORY AND LITERATURE REVIEW

Within the last ten years, the development of highly sophisticated analytical techniques for the detection of pesticide residues has been explosive.¹ These refinements have now led to fairly accurate estimates as to the extent of pesticide pollution in nature and the literature substantiating the persistence of these toxicants is voluminous.

Woodwell, in 1967, found DDT residues as high as 32 pounds per acre in a marsh on the south shore of Long Island, New York. The marsh had received extensive applications of the insecticide for twenty years. Appreciable amounts of DDT have also been detected in many organisms including plankton, bass, robins, dolphins, penguins, trout, and man.² Duggon and Weatherwax, in 1967, were able to identify residues of chlorinated hydrocarbon insecticides in a wide variety of food

¹Samuel D. Faust and Irwin H. Suffet, "Recovery, Separation, and Identification of Organic Pesticides from Natural and Potable Waters," Residue Reviews, XV (1966), 44-116; Francis A. Gunther, "Advances in Analytical Detection of Pesticides," Scientific Aspects of Pest Control, Publication 1402 of the National Academy of Sciences--National Research Council (Washington, D. C.: National Academy of Sciences, 1966), pp. 276-302.

²George M. Woodwell, "Toxic Substances and Ecological Cycles," Scientific American, CCXVI (March, 1967), 24-31.

classes including dairy products, meat, fish, vegetables, fruits, oils, and fats. The food samples were obtained from many major cities and DDT and its analogues, dieldrin, lindane, and heptachlor epoxide accounted for 85% of the total chlorinated pesticides found. All of the residues were below the limits set for acceptable daily intake by the World Health Organization and United Nations Committees.¹ The per cents of technical aldrin, chlordane, endrin, heptachlor, dilan, isodrin, BHC, and toxaphene remaining in Congaree sandy loam soil after fourteen years were found by Nash and Woolson, in 1967, to be 40, 40, 41, 16, 23, 15, 10, and 45, respectively.² Wiese and Basson, in 1966, mixed DDT, chlordane, heptachlor, dieldrin, and aldrin with different soil types and measured the rates of degradation and leaching monthly for twenty-four months. The order of residuality was found to be, in decreasing order, DDT, dieldrin, chlordane, aldrin, and heptachlor; with no differences occurring among the soil types.³ A

¹R. E. Duggon and Weatherwax, "Dietary Intake of Pesticide Chemicals," Science, CLVII (September, 1967), 1006-1010.

²Ralph G. Nash and Edwin A. Woolson, "Persistence of Chlorinated Hydrocarbon Insecticides in Soils," Science, CLVII (August, 1967), 924-927.

³I. H. Wiese and N. C. J. Basson, "The Degradation of Some Persistent Chlorinated Hydrocarbon Insecticides Applied to Different Soil Types," South African Journal of Agricultural Science, IX (December, 1966), 945-969.

review compiled by Edwards, in 1966, listed the average persistence of six chlorinated hydrocarbon insecticides based on all available data at the time of the review. He found less than 1% aldrin, chlordane, and heptachlor remained in the soil after four to six years, while lindane, dieldrin, and DDT were recoverable for up to twelve years in concentrations very near 5%.¹ Alexander, in 1965, concluded that, while chlorinated hydrocarbon insecticide concentrations do, in fact, decrease in a given soil, complete disappearance of the insecticides may never be realized. He based this concept on the fact that no data is available which shows complete elimination of insecticides such as toxaphene, heptachlor, aldrin, dieldrin, DDT, BHC, and chlordane from soils sampled over many years. The data reviewed by Alexander showed that after twelve years, an analysis of the soil could still detect residues of chlordane.²

The specific reasons for pesticide persistence are, for the most part, unknown. However, such molecular recalcitrance clearly indicates that biological and nonbiological mechanisms associated with normal

¹C. A. Edwards, "Insecticide Residues in Soils," Residue Reviews, XIII (1966), 83-132.

²M. Alexander, "Persistence and Biological Reactions of Pesticides in Soils," Soil Science Society of America, Proceedings, XXIX (January, 1965), 1-7.

degradative and detoxifying processes are essentially inoperative.¹ The nonbiological factors associated with pesticide disappearance include volatilization due to application and cultivation, absorption to particulate matter, desorption by rainfall and consequent leaching, photodecomposition, spontaneous breakdown, and non-enzymatic chemical reactions such as associated with pH changes.² Frequently, however, the basic mechanism of pesticide breakdown in a given environment is enzymatic or biological.³ Thus, persistence of noxious pesticides may, in many cases, be attributable to the inability of these organisms to destroy a given compound. Since this study concerns only the enzymatic phase of decomposition, nonbiological factors will be mentioned only with reference to their effects on biological degradation.

Theoretically, recalcitrance of pesticides toward microbial degradation is a function of either the chemical itself or the environment. Alexander, in 1965, expressed the opinion that recalcitrance of pesticides was

¹Edwards, op. cit., pp. 120-121.

²M. Alexander, "Biodegradation; Problems of Molecular Recalcitrance and Microbial Fallibility," Advances in Applied Microbiology, VII (1965), 35-77; Edwards, op. cit., pp. 118-119; P. C. Kearney, et al., "Behavior and Fate of Chlorinated Aliphatic Acids in Soils," Advances in Pest Control Research, VI (1965), 1-30.

³Edwards, op. cit., p. 119.

attributable to environmental or microenvironmental conditions not conducive to microbial growth and to compounds either totally or partially resistant to biodegradation under all environmental conditions. Environmental conditions favoring molecular recalcitrance include; anaerobiosis, deposition of metabolizable compounds in microenvironments physically impenetrable by microorganisms, and, of particular interest to this study, adsorption of either the substrate or enzymes on colloids such as silica. Other environmental factors suggested by Alexander were the coating or encrustation of compounds with nondegradable substances, absence of essential growth factors, unfavorable environmental conditions such as extremes in pH and low moisture, and vertical substrate movement into lower soil horizons not inhabited by microorganisms. All of these mechanisms would thus afford protection from decomposition to potentially degradable compounds. The chemical factors listed by Alexander suggest that many compounds, either totally or partially, resist degradation no matter how favorable the environmental conditions. He related this phenomenon with chemical configurations such as terminal quaternary groups, nonalkyl substituents, extensive branching of aliphatic moieties, and to a physiological inadequacy due to either the lack of proper enzymes

or to impenetrable substrates in the presence of organisms possessing the necessary, but intracellular, enzymes.¹

Edwards, in 1966, also summarized factors thought to be associated with pesticide recalcitrance and came to similar, though less exhaustive, conclusions.² Okey and Bogen, in 1965, suggested that the reason many chlorinated hydrocarbon insecticides resist metabolic attack is because of the presence of chlorine atoms on the aromatic rings. He theorized that since chlorine atoms are highly electrophilic, they inhibit the initial enzymatic attack on the ring which is also thought to be electrophilic in nature. It was also concluded that three or more chloride ions on a ring preclude metabolic attack, and, in the case of the dieldrin-like compounds, the chlorides are present in such a fashion that degradation or detoxification is not possible.³

Fortunately, many pesticides are subject to enzymatic attack in nature and the decomposition may take two pathways. Most compounds are either detoxified,

¹M. Alexander, "Biodegradation; Problems of Molecular Recalcitrance and Microbial Fallibility," Advances in Applied Microbiology, VII (1965), 35-77.

²Edwards, op. cit., pp. 116-121.

³Robert W. Okey and Richard H. Bogen, "Apparent Involvement of Electronic Mechanisms in Limiting Metabolism of Pesticides," Journal of the Water Pollution Control Federation, XXXVII (May, 1965), 692-712.

partially decomposed, or wholly utilized, thus rendering the originally noxious pesticide non-toxic to the environment. Alternatively, however, some pesticides are converted to even more toxic compounds by the degradative process.¹

The first comprehensive review of the role of microorganisms in hydrocarbon degradation was the now classic study published by ZoBell in 1946. ZoBell suggested that all kinds of gaseous, liquid, and solid hydrocarbons in the aliphatic, olefinic, aromatic, and naphthenic series were susceptible to oxidation by microorganisms, provided the hydrocarbons are properly dispersed.² This concept of the ubiquitous microbial world being omnipotent in its degradative powers led to many investigations concerning the action of microorganisms on various natural and synthetic organic products. Much work has been done to elucidate the various metabolic pathways of herbicide decomposition, but little knowledge is available concerning the degradation of insecticides. Kiigemagi, et al., in 1958, stated that the decline of aldrin, dieldrin, and

¹M. Alexander, "Persistence and Biological Reactions of Pesticides in Soils," Soil Science Society of America, Proceedings, XXIX (January, 1965), 1-7.

²C. E. ZoBell, "Action of Microorganisms on Hydrocarbons," Bacteriological Reviews, X (March-June, 1946), 1-49.

heptachlor couldn't be associated with soil microorganisms or with physical or chemical phenomenon.¹ In 1959, Lichtenstein and Schulz found that aldrin could be converted to dieldrin in soils held at physiological temperatures.² In later studies, they observed that the aldrin to dieldrin transformation was correlated with bacterial growth and was negligible in frozen soils and in autoclaved soils.³ Thus, it seemed that epoxidation of aldrin in soils was a function of microorganisms. Bollen, in 1960, reviewed the existing literature concerning pesticide degradation and observed that no conclusive evidence had yet been compiled to show that soil microorganisms were responsible for chlorinated hydrocarbon insecticide decomposition.⁴ In 1963, Martin

¹Ulo Kiigemagi, "Biological and Chemical Studies on the Decline of Soil Insecticides," Journal of Economic Entomology, LI (April, 1958), 198-204.

²E. P. Lichtenstein and K. R. Schulz, "Breakdown of Lindane and Aldrin in Soils," Journal of Economic Entomology, LII (February, 1959), 118-124.

³E. P. Lichtenstein and K. R. Schulz, "Persistence of Some Chlorinated Hydrocarbon Insecticides as Influenced by Soil Types, Rates of Application, and Temperature," Journal of Economic Entomology, LII (February, 1959), 124-131; E. P. Lichtenstein and K. R. Schulz, "Epoxidation of Aldrin and Heptachlor in Soils as Influenced by Autoclaving, Moisture, and Soil Types," Journal of Economic Entomology, LIII (April, 1960), 192-197.

⁴Walter B. Bollen, "Interactions Between Pesticides and Soil Microorganisms," Annual Review of Microbiology, XV (1961), 69-92.

also stated that microorganisms may not be the primary factor involved in the disappearance of aldrin, BHC, chlordane, dieldrin, and heptachlor from the soil, but that instead, loss of such compounds was probably due to volatilization. He was of the express opinion, however, that the vast majority of organic pesticides, or products of their partial degradation, are subject to microbial decomposition.¹ Recently, Baush and Malsumura described the role of Pseudomonas melophthora, the symbiote of the apple maggot (Rhagoletis pomonella), in the partial degradation of carbaryl, DFP, diazinon, dichlorovas, dieldrin, and parathion.² Whole cells or cell free extracts of Aerobacter aerogenes have been shown to effect the in vitro degradation of DDT to at least seven metabolites and a possible catabolic pathway has been proposed.³

Thus, microorganisms are capable of degrading, at least partially, some of the so called recalcitrant

¹J. P. Martin, "Influence of Pesticide Residues on Soil Microbiological and Chemical Properties," Residue Reviews, IV (1963), 96-129.

²G. Mallory Boush and Fumio Malsumura, "Insecticidal Degradation by Pseudomonas melophthora, the Bacterial Symbiote of the Apple Maggot," Journal of Economic Entomology, LX (August, 1967), 918-920.

³Gary Wedemeyer, "Dechlorination of 1,1,1-Trichloro-2,2-bis(p-chlorophenyl) ethane by Aerobacter aerogenes," Applied Microbiology, XV (May, 1967), 569-574.

chlorinated hydrocarbon insecticides. The extent of such degradative processes in nature, however, remains an unexplored facet in the entire picture of pesticide degradation. Also, to date, this author is unaware of any reports suggesting that chlordane is, in any way, affected by soil microorganisms. There are, however, a few isolated cases of chlordane metabolites being recovered from mammals.¹

Although this study attempts to clarify the in vitro effect of one microorganism on a pesticide, the influences of these compounds on the environment warrent some discussion since it is these influences which have caused so much concern regarding pesticides.

It has been assumed by many authorities that any toxic substance introduced into the environment will upset the delicate equilibrium existing between the microbial populations of a given ecosystem. Since soil fertility is dependent upon this homeostatic mechanism of the microbiota, it follows that introduction of a pesticide into the soil would upset this dynamic balance.

¹N. H. Poonawalla and F. Karte, "Metabolism of Insecticides. VII. Excretion, Distribution, and Metabolism of α -Chlordane by Rats," Life Science, III (December, 1964), 1497-1500; G. Ludwig, "Isolation and Identification of Metabolites of Some Chlorinated Insecticides and Their Detection by Analytical Methods," Radioisotopic Detection of Pesticide Residues, Proceedings, (1965), 49-58.

Audus, in 1964, proposed four different ways in which herbicides might affect microbial interrelationships, and these generalizations are applicable to insecticides.¹ The first possibility is that pesticides may act as metabolic inhibitors, blocking some essential anabolic or catabolic step common to many organisms. A specific example of this type of inhibition is that DDT or heptachlor completely inhibits the activity of lactate dehydrogenase in vitro.² Another possibility proposed by Audus is that a pesticide might be selectively toxic to a particular group of microorganisms which play an important role in maintaining soil fertility. The third microorganism-pesticide interaction conceived by Audus is pesticides acting to stimulate the growth of a particular population. This could occur directly by the pesticide acting as a carbon source for a group of microorganisms. It could also occur indirectly as exemplified by the toxicity of a pesticide towards a group of microorganisms which synthesize a nutrient required by some, but not all, microorganisms. Pesticide tolerant organisms, dependent on an extraneous source of the nutrient, would be

¹L. J. Audus (ed.), The Physiology and Biochemistry of Herbicides (New York: Academic Press, 1964), pp. 190-192.

²Carl R. Sova, "Lactate Dehydrogenase Activity: Effect in vitro of Some Pesticidal Chemicals," Science, CLIV (December, 1966), 1662.

inhibited along with the nutrient-producing, pesticide-sensitive microbes. Thus, pesticide-tolerant organisms, capable of synthesizing this hypothetical nutrient, would become the dominant biotype. These organisms could then be either harmful or beneficial to soil fertility. Finally, as suggested by Audus, it is possible that nutrients released from dead, pesticide-sensitive microorganisms would stimulate the growth of pesticide-tolerant heterotrophs, not necessarily capable of utilizing the pesticide as a carbon and energy source.¹

Many attempts have been made to demonstrate deleterious effects, such as those suggested by Audus,² of pesticides on the soil microbiota. However, for the most part, these attempts have been unsuccessful. Most investigations have concerned themselves with total bacteria and fungi counts, ammonification, nitrification, nitrogen fixation, and oxygen consumption. Audus stated that most herbicides, applied at the recommended rates, effect few permanent changes in the homeostatic mechanisms of the soil microflora.³ Whiteside and Alexander, in 1960, found that herbicides applied at the rates recommended for weed control had no effect on the soil

¹Audus, loc. cit.

²Ibid.

³Ibid., p. 191.

microflora in reference to oxygen consumption and deposition of carbonaceous materials.¹ Bartha, et al., in 1967, studied the influence of twenty-nine pesticides on soil microbes as a function of CO₂ production and nitrification. He and his associates were unable to demonstrate any significant changes in the two processes as a result of chlorinated hydrocarbon insecticide application.² Eno, et al., in 1964, evaluated the effects of six chlorinated hydrocarbon insecticides on soil microorganisms in terms of soil nitrate production, CO₂ evolution, relative numbers of fungi and bacteria, and relative rate of urea hydrolysis. An analysis of variance confirmed that the compounds DDT, lindane, toxaphene, chlordane, aldrin, and dieldrin applied at two to four times the recommended rates were not harmful to the microbial population.³ The effects of DDT, aldrin, chlordane, and BHC on available soil nutrients and microorganisms were observed by

¹Jean S. Whiteside and M. Alexander, "Measurement of the Microbiological Effects of Herbicides," Weeds, VIII (April, 1960), 204-213.

²Richard Bartha, et al., "Stability and Effects of Some Pesticides in Soil," Applied Microbiology, XV (January, 1967), 67-75.

³C. F. Eno, et al., "The Effects of Soil Applications of Six Chlorinated Hydrocarbon Insecticides on Vegetable Crops and Soil Microorganisms," Florida State Horticultural Society, Proceedings, LXXVII (1964), 223-229.

Pathak, Shankar, and Awasthi in 1961. Toxic effects on the soil microflora at rates of application up to 100 pounds per acre were not demonstrable. It was shown, however, that the number of bacteria increased as did nitrogen fixation, nitrification, and available phosphorous.¹

Generally, insecticide applications produce few, if any, deleterious effects on plants. Eno, et al., in 1964, treated various vegetable crops with DDT, lindane, toxaphene, chlordane, aldrin, and dieldrin at rates of two to four times those recommended for insect control for a period of five years. No yield reduction was noted on any of the crops except for a decrease in corn and tomato yields resulting from DDT application. Yields returned to normal when the use of DDT was discontinued.² Frear, in 1955, reported that chlordane has been found to be toxic to germinating seeds.³

Insecticides generally produce more demonstrable effects on the animal kingdom, not to mention the

¹A. N. Pathak, Hari Shankar, and K. S. Awasthi, "Effect of Some Pesticides on Available Nutrients and Soil Microflora," Journal of the Indian Society for Soil Science, IX (September, 1961), 197-200.

²Eno, et al., loc. cit.

³Donald E. H. Frear, Chemistry of the Pesticides (New York: D. Van Nostrand Co., Inc., 1955), p. 59.

toxicity towards their target organisms, the arthropods. It has been reported that insecticide residues have, in many ecosystems, reached levels that are catastrophic for fish populations and carnivorous birds.¹ Odum, in 1959, reported that, next to insects, fish and amphibians are most vulnerable to chlorinated hydrocarbon insecticide poisoning with birds and mammals being more tolerant.² Frear found that chlordane has an acute LD₅₀ for rats of 225 to 250 mg/kg, and, when fed at low levels in chronic toxicity tests, it causes severe liver damage.³ Chlordane has also been shown to be cytotoxic to tissue cultures by Gabliks and Friedman in 1965. Chang liver and He La cell cultures exhibited inhibition of cell growth, changes in cellular morphology, and, in some cases, destruction of the cultures occurred. The LD₅₀ and LD₁₀ reported by Gabliks and Friedman were 10 and 5 µg/ml, respectively.⁴

The hazards presented by pesticides for ecosystem

¹George M. Woodwell, "Toxic Substances and Ecological Cycles," Scientific American, CCXVI (March, 1967) 24-31.

²Eugene P. Odum, Fundamentals of Ecology (second edition; Philadelphia: W. B. Saunders Co., 1959), p. 425.

³Donald E. H. Frear, Chemistry of the Pesticides (New York: D. Van Nostrand Co., Inc., 1955), p. 59.

⁴Janis Gabliks and Leo Friedman, "Responses of Cell Cultures to Insecticides," Society for Experimental Biology and Medicine, Proceedings, CXX (October, 1965), 163-171.

dynamics are much more insidious than for individual organisms and are also less understood. Since consideration of the complexities of pesticide pressures on ecosystems is beyond the scope of this study, the reader is referred to the works of Alexander, Barret, Hunt, and Woodwell.¹

¹M. Alexander, "Biodegradation; Problems of Molecular Recalcitrance and Microbial Fallibility," Advances in Applied Microbiology, VII (1965), 35-77; Gary W. Barrett, "The Effects of an Acute Insecticide Stress on a Semienclosed Grassland Ecosystem," (unpublished Ph.D. thesis, The University of Georgia, Athens, 1967); Eldridge G. Hunt, "Biological Magnification of Pesticides," Scientific Aspects of Pest Control, Publication 1402 of the National Academy of Sciences-National Research Council (Washington, D. C.: National Academy of Sciences, 1966), pp. 251-262; Woodwell, loc. cit.

CHAPTER III

MATERIALS AND METHODS

Most insecticides which are applied to crops, lawns, cities, and even livestock eventually reach the soil, either directly or indirectly. Therefore, it seemed reasonable that organisms capable of degrading or detoxifying insecticides might be present in soils which had received extensive applications of pesticides, ie., fungicides, herbicides, and insecticides. Such an area is the European Corn Borer Research Station in Ankeny, Iowa. One particular test plot of the Research Station, designated the "Screening Area" (SA), was chosen as the source of soil samples for the investigation. This area is used to determine the effectiveness of newly developed commercial insecticides in controlling the European corn borer. The SA is approximately a five acre plot of Clarion silt loam and is planted in corn each year. Records indicate that, over the years, the SA has been contaminated with almost every type of pesticide known. In addition, a yearly fertilizer program has been followed consisting of a nitrogen preplant of approximately 90 lb actual N per acre applied as 33% NH_4NO_3 . This was followed by a side dressing of 100 lb per acre of 6-24-24 at the time of planting. On

the day the soil samples were obtained (April 28, 1967) the area had just received an application of 1 lb actual heptachlor (1,4,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene¹) and 1 lb actual atrazine per acre.

Three random samples (SA-1, SA-2, and SA-3) were taken from the SA and the samples were placed in "Dispo²-Cups" and frozen until needed. Circumstances prevented immediate use of the samples and thus they were held in this frozen state (approximately -15 C) for about three months until experimentation was resumed.

When ready for use, the three samples were thawed, and each was divided into two lots (SA-1A and SA-1B, SA-2A and SA-2B, and SA-3A and SA-3B). One lot of each sample (SA-1A, SA-2A, and SA-3A) was brought to apparent dryness by being held in an oven at 65 C for three days. Then, each was triturated with a mortar and pestle and screened with a common flour sifter having a mesh size of 1.5 mm. A device of the authors own design (Figure 1) was used to determine the water holding capacity of each sample. The device employed was constructed by fitting a small plastic glass with a screen of small mesh after

¹ Name conforms to International Union of Physical and Applied Chemistry nomenclature.

² Registered trademark, distributed by Scientific Products, a division of American Hospital Supply Corp., Evanston, Illinois.

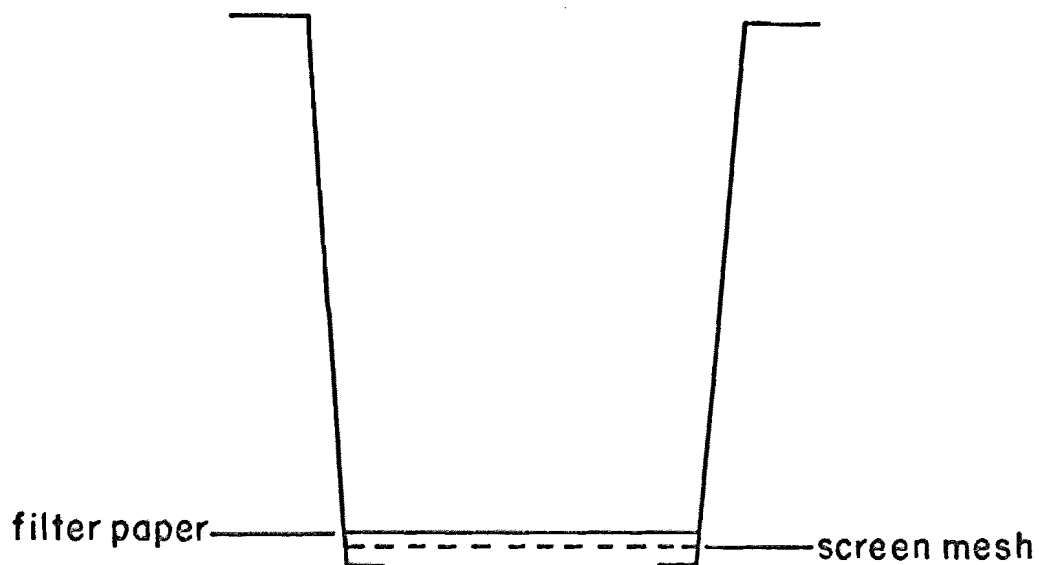


Figure 1. Device used to determine the maximum water holding capacity ($WHC_{100\%}$) of a dried soil sample. Drawn to exact size.

having removed the bottom of the glass. A filter paper disc, No. 1, was placed inside the cup, on top of the screen. The disc was then moistened so that a tight fit was obtained. A 5.0 g sample of dried, screened soil was then placed in the cup and the cup plus soil was weighed. An excess of water was poured over the soil and allowed to settle. When water ceased to drain from the bottom of the cup, the cup was reweighed and it was assumed that the tare was a rough approximation of the maximum water holding capacity of the 5.0 g sample. The maximum water holding capacity ($WHC_{100\%}$) of each sample was determined in triplicate and, when calculated, was found to be 0.69 ml/g for all of the samples. The other lot of each sample (SA-1B, SA-2B, and SA-3B) was then brought to apparent dryness by holding these samples for 24 hours in an incubator set at 35 C. A lower temperature was chosen for these samples in order to prevent any major changes in the soil such as protein denaturation and death of the soil microflora. These lots were then ground and screened in the manner perviously described in order to obtain the 1.5 mm fraction. These dried, screened samples of the second lot of each sample were then placed in a dessicator to be used for respiration studies and for a microorganism source.

Technical grade chlordane was supplied for the

study by Velsical Chemical Corporation and, with the exception of density, the chemical and physical properties for the product were reported by Bowery in 1964. The technical grade material is a viscous, amber liquid having a boiling point of 175 C at 2.0 mm Hg. Chlordane is insoluble in water, but is miscible with many aliphatic and aromatic hydrocarbons, esters, ethers, ketones, and most organic solvents. Its vapor pressure is 1×10^{-5} mm Hg at 25 C and it has a viscosity of 69 poises at 25 C. Chlordane may be dechlorinated, with a trace of iron catalyzing the reaction, to yield a non-toxic product by the loss of hydrogen chloride. The density of this particular sample was found to be 1.640 g/ml. Technical chlordane is known to be a mixture of 60-75% alpha- and beta-chlordane and 25-40% heptachlor, hexachlor, and Trichlor 237; these compounds being separable by either paper, column, or gas chromatography.¹

Suspension of the chlordane for respirometer studies was achieved by employing the method of Harding. One gram (0.61 ml) of chlordane was introduced into a 1000 ml volumetric flask and to this was added 0.10 ml

¹T. G. Bowery, Chlordane, (Vol. II of Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives, ed. Gunter Zweig. New York: Academic Press, Inc., 1964), pp. 50-51.

of Triton¹ X-155 (alkyl phenoxy polyethoxy ethanol), an emulsifier supplied for the purposes of this investigation by Rohm and Haas Company. This was followed by 100 ml acetone and sufficient distilled water to fill the volumetric flask to the mark.² This procedure yielded a fairly stable emulsion with a concentration of 1000 mg/liter of the active ingredient. The suspension was then serially diluted to concentrations of 100 and 10 mg/liter of the insecticide. Future reference to a chlordane-acetone-Triton X-155 mixture will be X mg/liter CAT.

Control solutions were also prepared for use in the respirometer. Ten milliliters of acetone were added to 90 ml of water and this solution was serially diluted to 1.0 and 0.1% (v/v) concentrations of acetone. This procedure yielded three solutions with concentrations of acetone equivalent to that of the acetone concentrations in the 1000, 100, and 10 mg/liter chlordane suspensions. A second control solution was then prepared containing acetone and Triton X-155. The procedure

¹Registered trademark, Rohm and Haas, Philadelphia, Pennsylvania 19105.

²James A. Harding, "Laboratory Screening of Crop Protection Chemicals on the European Corn Borer" (Ankeny, Iowa: United States Department of Agriculture, Agricultural Research Service, Corn Borer Investigation, 1967), p. 1. (Mimeographed)

employed was that described for the acetone-water control with one exception. The source of acetone was a mixture of 100 ml acetone and 0.10 ml Triton X-155. This step was required due to the viscous nature of Triton X-155 which made it difficult to quantitatively pipette any volume less than 0.1 ml. Ten milliliters of this mixture was then added to the 90 ml of water and this acetone-emulsifier-water mixture was then serially diluted to yield the three desired solutions of 100, 10, and 1.0 mg/liter Triton X-155. The final dilution of each suspension was arbitrarily chosen for use in the respirometer study (Table 1).

Each SA soil sample was measured for an increase in respiration rate in the presence of chlordane as compared to control soils without the insecticide. A Gilson Respirometer, Model GP-14, was used for the study and the flasks were prepared after the method of Chase and Gray.¹ The flasks were of the single side-arm Warburg type and each was thoroughly cleaned, rinsed, and dried before use. A 2.0 g sample of the dried, screened soil from the second lot of each was placed in the main chamber of the flask. At the same time this operation

¹F. E. Chase and P. H. H. Gray, "Application of the Warburg Respirometer in Studying Respiratory Activity in Soil," Canadian Journal of Microbiology, III (March, 1957), 335-349.

TABLE I

FLASK-SUBSTRATE DISTRIBUTION AND SUBSTRATE
CONCENTRATIONS OF THE PRIMARY ISOLATION
RESPIROMETER STUDY

Flask	Soil (grams)	Substrate	Substrate (ml)	Substrate Concentration ¹
1	2.0	H ₂ O	0.82 ²	-
2	2.0	CAT ³	0.82	10C
3	2.0	A	0.82	0.1%A
4	0.0	H ₂ O	1.00	-
5	2.0	H ₂ O	0.82	-
6	2.0	CAT	0.82	10C
7	2.0	TA	0.82	1T
8	2.0	TA	0.82	1T
9	2.0	A	0.82	0.1%A
10	2.0	A	0.82	0.1%A
11	0.0	H ₂ O	1.00	-
12	2.0	H ₂ O	0.82	-
13	2.0	TA	0.82	1T
14	2.0	CAT	0.82	10C

¹Stated in mg/liter except for acetone which is expressed in % by volume

²This value is equal to WHC_{60%} for 2 g soil

³C=chlordan, A=acetone, T=Triton X-155

was performed, a small amount of soil (approximately 0.5 g) from each sample was placed on chlordane agar slants and incubated at 30 C until growth was obtained. This enrichment technique was an attempt to insure preservation of any organism(s) capable of utilizing chlordane that might be lost during the respirometer studies. The chlordane agar slants were prepared by adding 0.1 g chlordane to 1.0 liter of a Czapek Dox Agar base in which the saccharose had been omitted.¹ This chlordane Czapek Dox agar was autoclaved at 15 psi for 15 minutes, dispensed into sterile 145x18 mm screw cap tubes, and the tubes were allowed to solidify in the slanted position. Prior to pouring the chlordane Czapek Dox agar into the tubes, it was thoroughly agitated to insure that the hydrophobic chlordane was emulsified. The slants were then incubated at 30 C until heavy growth was obtained. The soil in each flask was then brought to 60% water holding capacity (WHC_{60%}), as recommended by Alexander, by adding the appropriate amount (0.41 ml/g) of substrate or water (Table 1).² Next, 0.2 ml of 3% KOH was added to the

¹Difco Laboratories, Inc. Difco Manual (ninth edition; Detroit: Difco Laboratories, Inc., 1953), p. 245.

²Martin Alexander, Introduction to Soil Microbiology (New York: John Wiley and Sons, Inc., 1961), p. 25.

center well of each flask and the rim of the well coated with petrolatum. Each center well was then fitted with "paddle-shaped" pieces of filter paper. The blade of the paddle was equal in width to the inner diameter of the well and the narrow handle extended upwards without touching the rim of the well or the above standard taper stopper of the volumeter. This "paddle" had two functions. First, it retarded creeping of the KOH caused by dissolution of moisture from the atmosphere of the flask. Secondly, it afforded a greater surface area of KOH for CO_2 dissolution. The neck of each flask was coated with a liberal amount of petrolatum and the flasks were then connected to the volumeter. Aseptic techniques were not practiced during the screening procedure, nor were any of the flasks or substrates sterilized. These steps were unnecessary since the technique was used only as a screening procedure for the isolation of an organism(s) capable of degrading chlordane. All fourteen flasks were employed to screen each soil sample, with two of the flasks containing only 1.0 ml of water. These two flasks acted as thermobarometers, reflecting any temperature or pressure changes that might have occurred in the closed system. The remaining twelve flasks were divided into four sets as follows: (1) three flasks containing soil and water,

(2) three flasks containing soil and 0.1% (v/v) acetone, (3) three flasks containing soil and 1.0 mg/liter Triton X-155 in 0.1% (v/v) acetone, and (4) three flasks containing soil and 10 mg/liter CAT. The flasks were randomized in the water bath (Table 1) and they were allowed to equilibrate for one hour the first day. On each successive day, equilibration was unnecessary since the flasks were maintained in the water bath at all times. At the beginning of each run, the operating and gassing manifold valves were closed, the operating valves being closed simultaneously via the master valve lever. Agitation of the flasks afforded no advantage since the soil-substrate combination was of a solid nature. The digital micrometers were read as required by movement of fluid in the manometers during a four to ten hour period each day. The readings obtained were expressed in terms of microliters O_2 consumed/hour per 2.0 g soil after being corrected for standard pressure and changes reflected by the thermobarometers.

Those flasks which reflected a higher respiratory activity than the controls containing only soil and water were used as the source of microorganisms. A small amount of soil (approximately 0.1 g) was removed aseptically from the flasks showing a high respiratory activity in the presence of chlordane by using a sterile,

small spatula. This soil was then subcultured on Czapek Dox agar slants in which 1.0 liter of 10 mg/liter CAT was substituted for the 1.0 liter of water and the saccharose was omitted. The agar was then dispensed in 145x18 mm screw cap tubes and sterilized in an autoclave at 15 psi for 15 minutes. These subcultures were then placed in an incubator and held at 30 C until sufficient growth had occurred; a period of time usually lasting three to four days.

Growth from the slants was then aseptically transferred to 100 mg/liter CAT-Czapek Dox agar plates prepared as described for the CAT-Czapek Dox agar slants. The plates were streaked in order to isolate a pure culture of the organism(s) responsible for the increased respiratory activity. These plates were again incubated at 30 C until good growth was obtained. Isolated colonies were then subcultured on Bacto¹-Stock Culture Agar slants and 100 mg/liter CAT-Czapek Dox agar slants in order to maintain stock cultures of the isolants. The stock cultures were stored in a refrigerator at 5 C and subcultured on fresh media every month in order to ensure viability of the organisms. These stock cultures were then used as the organism source for the rest of the

¹Registered trademark, Difco Laboratories, Inc., Detroit, Michigan.

study.

The organism(s) isolated from the respirometer study just described was then subjected to a second respirometer study. This second run was an attempt to confirm the high oxygen consumption rates exhibited by the flasks containing the exogenous substrates during the first respirometer study. Three flasks were employed for this run and the remaining eleven volumeters were disconnected by closing the disconnect valves on each. A volume of water equal to the total volume of the eleven unused flasks was added to the reference flask. This procedure equalized the volume in the reference flask and the total volume of the three flasks, thus preventing a drastic change in the experimental flasks air pressure as a result of a small change in air pressure in an empty reference flask. The flasks were of the single side arm Warburg type and they were cleaned very thoroughly before use in an attempt to insure chemical cleanliness. The center well of each flask was then prepared as described for the first study. The main chamber of two of the three flasks received 1.8 ml of Czapek Dox basal broth medium and 0.2 ml of a culture suspended in, and washed from a 100 mg/liter CAT-Czapek Dox agar slant with, pH 7.2 Sorensen's phosphate buffer (M/15). To one of these

flasks was added 0.2 ml of a 10% (v/v) aqueous acetone solution and the other flask received 0.2 ml of a 1000 mg/liter CAT solution. Thus, excluding the contribution to the volume by the culture suspension, one flask contained 1% (v/v) acetone and the other 100 mg/liter chlordane. The third flask represented a control and contained 2.2 ml H_2O . The water bath was held at 30 C and the shaker was utilized due to the liquid nature of the culture. After an initial equilibration of one hour, the operating valves and gassing manifold valve were closed and readings were taken at two to six hour intervals as necessitated by movement of the manometer fluid level. The flasks were opened to the atmosphere at the end of each day and closed for the next days measurements without any further equilibration. Measurement periods for a day ranged from eight to fourteen hours. The resulting readings were corrected to standard pressure and for changes reflected by the control flask and expressed as microliters oxygen per hour per 2.2 ml culture at 30 C.

At this point, the question may be raised as to why different chlordane media were employed for various studies. The media changes merely reflected an evolution of techniques in an attempt to obtain: (1) a chlordane medium free of other carbon entities, (2) a medium of

homogenous phase, and (3) a medium capable of supporting luxuriant growth. However, this idealized medium is difficult to realize with hydrophobic hydrocarbons such as chlordane. Suspension of chlordane in Czapek Dox basal agar medium proved to be unsatisfactory as it was not possible to obtain homogenous dispersion of the chlordane. Emulsification of the chlordane in acetone and Triton X-155 prior to addition of the basal medium was also undesirable since this method contributes three potentially degradable carbon skeletons. Thus, subsequent to the respirometer studies, the method of choice for chlordane suspension in basal salts agar was the recently developed technique of Barvak, Alroy, and Mateles.¹ This procedure involved adhering the desired liquid hydrocarbon to an equal weight of colloidal silica in the presence of ethyl ether. The slurry was thoroughly mixed in a mortar and the ether was then allowed to evaporate. This powder was then incorporated into the agar medium quite easily and the hydrocarbon had little tendency to be released from the silica as a result of autoclaving. If, prior to solidification, the melted agar was shaken thoroughly, the powder remained dispersed throughout the medium and it

¹J. N. Barvak, Y. Alroy, and R. I. Mateles, "Incorporation of liquid hydrocarbons into Agar Media," Applied Microbiology, XV (July, 1967), 961.

imparted little opacity to the solidified agar (see appendix). This method of chlordane suspension will henceforth be referred to as CS (chlordane-silica) agar with the addition of necessary terms to define the type of basal salts used. Suspension of the hydrophobic chlordane in liquid media has proved more difficult and, as of this writing, a method satisfying all three criteria has not been found. Thus, the method of choice for preparing liquid chlordane media has been that of emulsifying the chlordane in an acetone and Triton X-155 mixture before incorporation into the basal salts solution. This procedure had many disadvantages. The chlordane had a tendency to settle out as a white precipitate unless the media was constantly agitated. In addition, the media was not translucent and it was difficult to ascertain whether or not growth had occurred as determined with the naked eye. Finally, in addition to chlordane, two other carbon sources were available for utilization.

In the latter stages of the study, a new basal medium was formulated by the author, and this basal salts solution was used for all studies performed subsequent to the isolation and characterization of the organisms. The basal salts solution was composed of 0.50 g ammonium sulfate, 0.50 g sodium nitrate, 0.10 g dibasic potassium phosphate, 0.004 g monobasic potassium

phosphate, 0.02 g magnesium sulfate, 0.02 g calcium sulfate, and 0.005 g ferrous sulfate present in one liter of distilled water (see appendix). The ratio between the monobasic and dibasic potassium phosphates very lightly buffered the medium at approximately pH 8.0. This basal medium satisfied two conditions, neither of which was satisfied by the Czapek Dox basal salts solution: (1) it supplied nitrogen for protein synthesis as both ammonium and nitrate ions, and, (2) it was virtually free of any chloride ion. These two requirements had to be met for two reasons. One organism isolated was incapable of reducing nitrate to the ammonium ion needed for amino acid synthesis. Secondly, a medium free of chloride ion was needed if an attempt was to be made to detect chloride ions liberated from the chlordane molecule in a culture. The basal salts was termed the chloride-free basal broth medium (CFBBM) or chloride-free basal agar medium (CFBAM) and will be referred to by the letters CFBBM or CFBAM as necessary.

In an attempt to determine which component of the CAT system was being utilized, the following media were prepared, with extreme caution being exercised to prevent contamination of the media with foreign carbon sources:

1. 100 mg/liter CS-Czapek Dox agar slants
2. 1% (v/v) acetone Czapek Dox agar slants with the

appropriate amount of sterilized 10% (v/v)
aqueous acetone added aseptically to auto-
claved Czapek Dox basal agar medium

3. 10 mg/liter Triton X-155 Czapek Dox agar slants
4. Basal Czapek Dox agar slants free of any carbon
source except the agar

The above were prepared with 1.0% Special Agar (Noble),
a highly washed and pure agar available from Difco
Laboratories Inc.. Loss of some acetone from the agar
was unavoidable due to the temperature of the melted
agar medium. However, the presence of a methyl ketone
in a small sample of the agar was ascertained by employing
a test for the detection of methyl ketones. The test was
based on a color reaction which occurs when methyl ketones
are treated with sodium nitroprusside and subsequently
acidified.¹ The four agar media were then inoculated
with a small amount of growth from a 48 hour 100 mg/liter
CS-Czapek Dox agar slant and incubated at 30 C.

Microscopic examination of the organisms was made
with a bright field scope to determine form, arrangement,
and Gram reaction. Phase contrast examination of a
hanging drop preparation revealed the size of the

¹Fritz Feigl (ed.), Spot Tests in Organic Analysis,
trans. Ralph E. Oesper (seventh edition; New York: Elsevier
Publishing Company, 1966), pp. 208-209.

organisms as measured with an ocular micrometer. The same hanging drop preparation was used to demonstrate motility. A hanging drop preparation examined with a phase contrast microscope was also employed to observe the pellicles formed by the organisms in liquid media. Leifson's flagella stain was employed to determine the type of flagellation.¹ A capsule stain was made as suggested by Fair. This capsule stain consisted of emulsifying a small drop of India ink in a loopful of culture, allowing the suspension to air dry without subsequent heat fixation, and counterstaining with crystal violet for thirty seconds.²

Biochemical tests were next performed in an attempt to characterize the organism(s) isolated from the respirometer studies. Unless stated otherwise, all media employed for the tests were obtained from Difco Laboratories, Inc.; Detroit, Michigan. Fermentation studies were run on the carbohydrates and carbohydrate derivatives acetone, ethanol, fructose, galactose, glucose, glycerol, lactose, maltose, mannitol, n-propanol, starch, sucrose, and xylose. The carbohydrates were present as

¹Society of American Bacteriologists, Manual of Microbiological Methods (New York: McGraw-Hill Book Company, 1957), p. 24-27.

³Jerrell F. Fair, personal communication.

1.0% concentrations in Bacto-Phenol Red Broth Base and the media was distributed in Durham fermentation tubes. In order to prevent complete volatilization of the acetone, and due to the fact that cellulose acetate membrane filters are soluble in this ketone, the following procedure was used to yield the required 1% acetone broth. Unsterile acetone was added to sterile water to make a 10% (v/v) acetone solution. The appropriate amount of this 10% aqueous acetone solution was then added to sterile Bacto-Phenol Red Broth Base thus yielding a 1.0% (v/v) acetone broth. Sterility of the acetone broth was verified by incubation at 28 C for 48 hours. The ability to oxidize glucose, lactose, and sucrose was examined according to the procedures described by Hugh and Leifson (see appendix).¹ Hydrogen sulfide production was assayed for by using Bacto-SIM medium and Bacto-Kligler Iron Agar. Nitrate reduction was detected by using Bacto-Nitrate Broth and powdered zinc was added to negative tests to determine whether or not nitrate was still present. Indole production was tested for by using Bacto-Tryptone broth enriched with 0.01% tryptophan. Bacto-M.R.-V.P. Medium was employed for the methyl

¹Rudolph Hugh and Einar Leifson, "The Taxonomic Significance of Fermentative Versus Oxidative Metabolism of Carbohydrates by Various Gram Negative Bacteria," Journal of Bacteriology, LXVI (July, 1953), 24-26.

red and Voges-Proskauer tests and citrate utilization was determined by using Bacto-Citrate Medium. Bacto-Tryptic Soy Agar enriched with 4.0% nonfat dry milk was the medium chosen to demonstrate casein hydrolysis and Bacto-Litmus Milk was utilized to test for litmus reduction and lactose fermentation. The ability to liquefy gelatin was investigated by stabbing Bacto-Nutrient Gelatin deeps. Urease was assayed for indirectly with Bacto-Urea Broth. Cytochrome oxidase production was demonstrated by the procedures of Gaby and Hadley as modified by Ewing and Johnson and by that of Kovacs (see appendix).¹ Anaerobic metabolism of arginine was determined by the method of Thornley and the presence of acetamide deamidase was demonstrated indirectly after the method suggested by Buhlmann, Visser, and Bruhin (see appendix).² Deamination of phenylalanine was investigated in the manner developed

¹P. R. Edwards and W. H. Ewing, Identification of Enterobacteriaceae (Minneapolis: Burgess Publishing Company, 1962), p. 251-252, citing International Bulletin of Bacterial Nomenclature and Taxonomy, Vol. X, p. 223. H. Kovacs, "Identification of Pseudomonas pyocyanea by the Oxidase Reaction," Nature, CLXXVIII (September, 1956), 703.

²Margaret J. Thornley, "The Differentiation of Pseudomonas From Other Gram Negative Bacteria on the Basis of Arginine Metabolism," Journal of Applied Bacteriology, XXIII (April, 1960), 37-52. X. Buhlmann, W. A. Vischer, and H. Bruhin, "Identification of Apyocyanogenic Strains of Pseudomonas aeruginosa," Journal of Bacteriology, LXXXII (November, 1961), 787-788.

by Ewing, Davis, and Reavis (see appendix).¹ Growth of the organisms in CFBBM to which 0.1% Bacto-Vitamin Free Casamino Acids had been added was observed.

The ability of the amino acids alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine to support growth when present as sole carbon sources in a chemically defined medium was determined. The amino acids were added to CFBBM as 0.01M concentrations and the resulting media was a clear broth, free from any sediment. The amino acid broths were then inoculated by removing a very small amount of growth from Bacto-Stock Culture agar slants. Growth was determined by observing for marked turbidity. Growth due to the introduction of a residual amount of complex substrate from the agar slants or to allelocatalysis was compensated for by recording as negative, any very small amount of particulate matter or turbidity present at the bottom of the tube which did not increase in amount with time. The final results were not recorded until the end of two weeks incubation due to the fact that enzyme systems

¹W. H. Ewing, Betty R. Davis, and R. W. Reavis, "Phenylalanine and Malonate Media and Their Use in Enteric Bacteriology," The Public Health Laboratory, XV (1957), 153-161.

responsible for amino acid degradation are many times inducible.¹

The limits of growth as a function of pH was determined by adding the required amount of dilute HCl and NaOH to Bacto-Tryptic Soy broth while measuring the pH with a pH meter. When the appropriate pH was reached, the broths were filter sterilized. A series of pH variables were thus prepared ranging from pH 3.5 to 11.0 with intervals of pH 0.5. Each tube of pH broth was then inoculated by introducing, from a sterile disposable pipette, two drops of a 48 hour Bacto-Tryptic Soy broth culture.

Growth, as a function of NaCl concentration, was also determined by preparing Bacto-Tryptic Soy broth with salt concentrations ranging from 0.5% to 7.0% NaCl. The salt concentration increased to the upper value at 0.5% intervals. Each tube of salt broth was then inoculated as described for the pH tolerance study.

Due to limited incubator facilities, no concentrated effort could be made to determine the exact limits of growth as a function of temperature and the exact optimum temperature. However, an approximation of the

¹William Burrows, Textbook of Microbiology (eighteenth edition; Philadelphia: W. B. Saunders Company, 1963), p. 168.

limitation on growth by temperature was realized by inoculating Bacto-Tryptic Soy broth and placing it in either a water bath or a refrigerator of previously determined temperature. A positive test consisted of growth occurring after three successive transfers of the culture at a particular temperature. During the course of the study, some idea was obtained concerning the optimum temperature of the organisms. However, this temperature preference is not critical and can vary five degrees either way with little effect on the rapidity or amount of growth.

Colonial morphology was described on Bacto-Tryptic Soy agar, Bacto-Blood Agar Base enriched with 5.0% defibrinated human blood, Bacto-Tryptic Soy Agar enriched with 4.0% nonfat dry milk, and 100 mg/liter CS-CFBAM with the agar being present as a 1.0% concentration. Pigment production was elicited on King's chromogenic agars A and B and also on Bacto-Tryptic Soy Agar supplemented with 10% egg white (see appendix).¹

The inoculum for all biochemical tests was, unless

¹Elizabeth O. King, Martha K. Ward, and Donald E. Raney, "Two Simple Media for the Demonstration of Pyocyanin and Fluorescin," Journal of Laboratory and Clinical Medicine, XLIV (August, 1954), 301-307. J. A. Garibaldi, "Media for the Enhancement of Fluorescent Pigment Production by Pseudomonas Species," Journal of Bacteriology, XCIV (November, 1967), 1296-1299.

otherwise indicated, growth from Bacto-Stock Culture agar slants. All tests were carried out at 30 C and incubated at this temperature for at least 48 hours depending on the test performed.

Tests were undertaken to describe optimum conditions for chlordane degradation to occur. Again, due to limited incubator facilities, the optimum temperature for growth could not be determined with any precision. However, during the course of the entire study, it was observed that approximately 28-30 C was the temperature at which the best growth occurred on all media, including the various chlordane media.

Growth in 100 mg/liter CAT-Czapek Dox broth as a function of pH was determined turbidometrically by using a Bausch and Lomb Spectronic¹ 20 colorimeter. The media was buffered at the required pH by using M/7.5 Sorensen's phosphate buffer mixtures. The M/7.5 K_2HPO_4 and M/7.5 KH_2PO_4 was combined in the proper volume ratios to obtain solutions buffered at the following pH values: 5.30, 6.24, 6.98, 7.38, and 8.04. One hundred milliliters of each of these buffer solutions was then added to 100 ml of 200 mg/liter CAT-double strength Czapek Dox basal broth medium. This procedure yielded five solutions of

¹Registered trademark, Bausch and Lomb, Rochester, New York.

100 mg/liter CAT-Czapek Dox broth buffered at the various pH values with a M/15 K_2HPO_4 - KH_2PO_4 mixture. Each solution was divided into two lots of 100 ml, placed in 250 ml Erlenmeyer flasks, stoppered with cotton plugs, and autoclaved for 15 minutes at 15 psi. Prior to inoculation of the media, small aliquots were aseptically withdrawn from each flask and the pH was checked with a pH meter. The ten flasks were found to have the following pH values: 5.15, 5.15, 6.15, 6.15, 7.10, 7.10, 7.50, 7.50, 7.90, and 7.90. One flask of each pH was then inoculated with a cell suspension from a 72 hour 100 mg/liter CAT-Czapek Dox broth culture of organisms grown in a shaker-water bath at a temperature of 28 C. The cells in the inoculum had been spun down, washed twice with sterile 0.85% saline, and resuspended in the sterile saline. The optical density of the inoculum was then determined, using the sterile saline as a blank and it was found to be 0.08 (O. D. at 570 millimicrons). This standardization of the inoculum was done so that the experiment could be duplicated. The other five flasks were not inoculated and served as sterile controls. The controls were used as blanks against which the inoculated broth of the corresponding pH was compared to follow growth as a function of turbidity. A determination of pH was also made on controls and inoculated broths in order that

any variation in pH between a control and corresponding culture could be detected. The ten flasks were placed in a 28 C shaker-water bath and, at 48 hours and daily thereafter, small samples were aseptically removed from each flask for pH and turbidity measurements. Also, at the end of four days, Gram stains were made on each inoculated flask and samples from all ten flasks were introduced into tubes of Bacto-Tryptic Soy Broth to determine whether or not viable cells were present in either the controls or the inoculated broths.

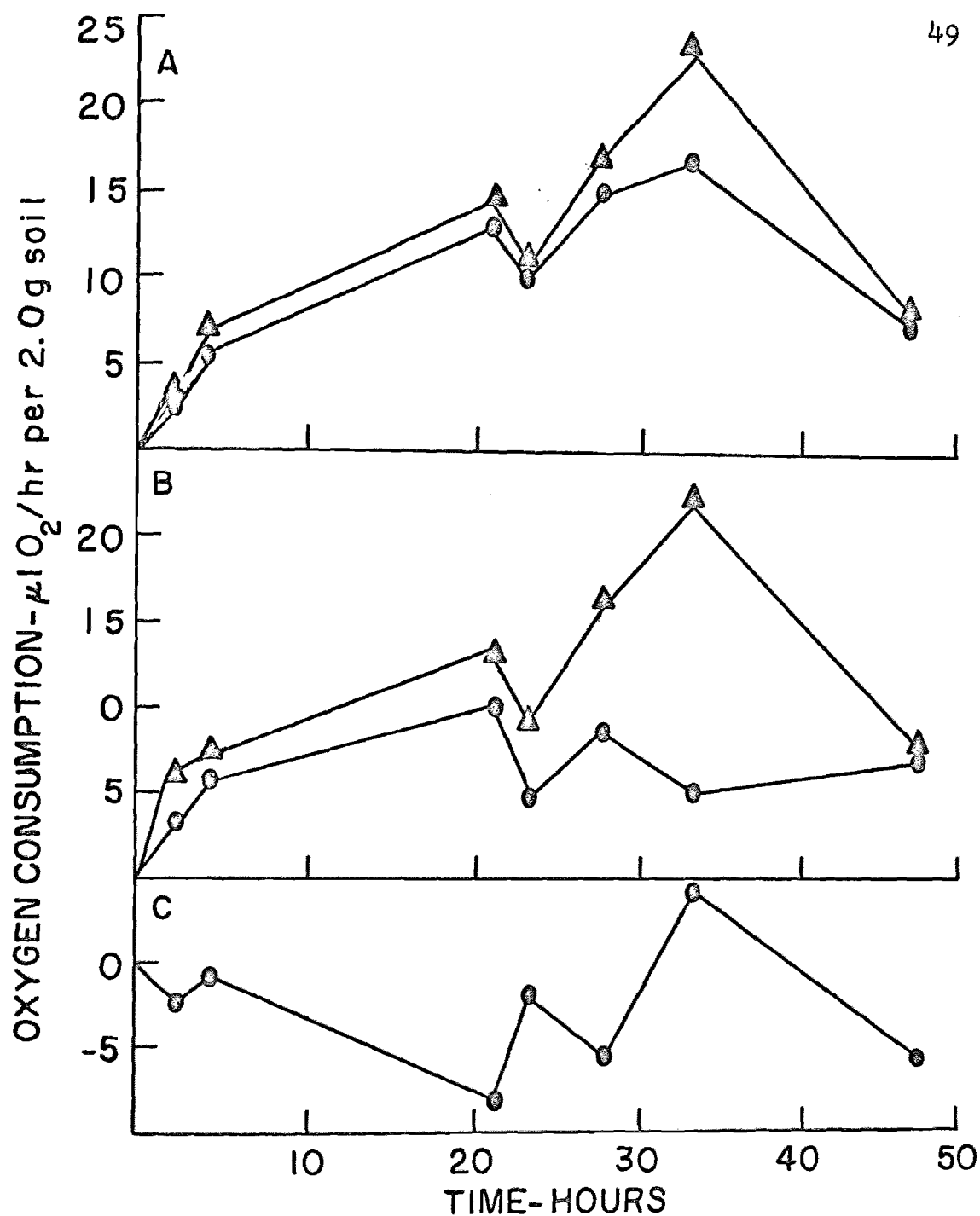
Growth in chlordane broth was then followed as a function of chlordane concentration. A 10,000 mg/liter CAT-CFBBM was prepared and this was serially diluted to 0.0001 mg/liter in CFBBM. The dilution series was dispensed in tubes and inoculated with a loopful of syneresis fluid from cultures grown on 100 mg/liter CS-CFBAM slants. The tubes were incubated in an incubator at 28 C. The growth was then followed turbidometrically with the Spectronic 20. An uninoculated tube of broth at the particular dilution corresponding to the inoculated broth being measured, was used as the blank.

CHAPTER IV

RESULTS

Growth was obtained by enrichment with a 100mg/liter chlordan-Czapek Dox agar slant into which the SA-2B and SA-3B mixture had been inoculated. A liberal amount of this growth was removed from the slant and streaked onto a 100 mg/liter CAT-Czapek Dox agar plate so as to obtain isolated colonies. The resulting colonies were very small, hard, and a glistening white in color. Examination of Gram stains performed on several colonies revealed that all of the colonies consisted of small Gram negative bacilli, occurring singly and in small chains. Subcultures were then made of these isolated colonies by inoculating one tube of a 100 mg/liter CAT-Czapek Dox agar slant and one tube of a Bacto-Stock Culture agar slant each with several colonies. These organisms were temporarily designated as "Isolant SA-2B:3B".

A preliminary screening of the SA-2B-SA-3B soil sample mixture with the respirometer revealed that those flasks having an exogenous substrate were respiring at rates significantly higher than those exhibited by the soil-water controls (Figure 2). A small amount of soil from the acetone containing flask (Flask No. 3) was removed and introduced into a 100 mg/liter CAT-Czapek

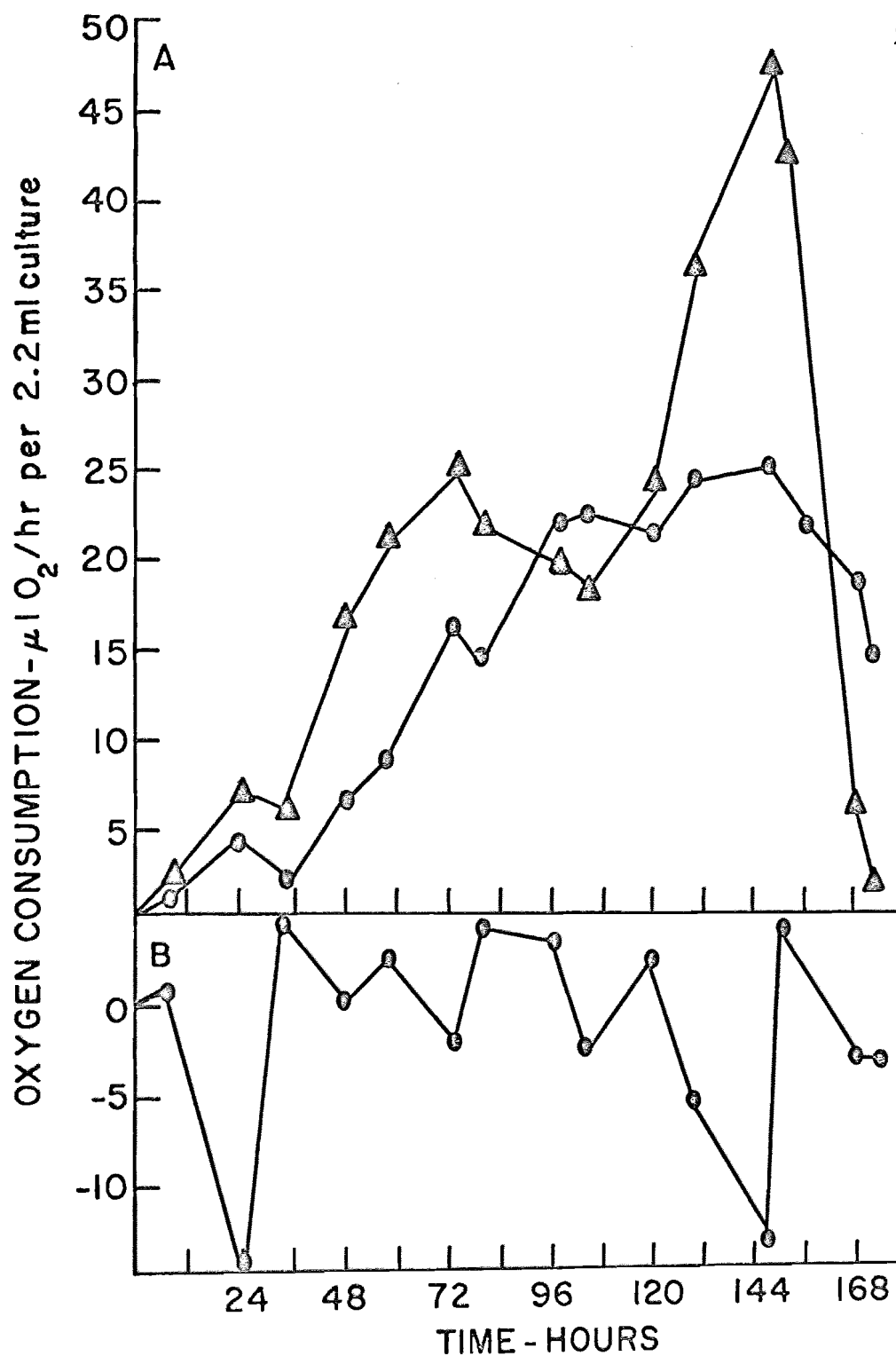


Figures 2A, B, and C. Mean oxygen consumption in $\mu\text{l O}_2/\text{hr}$ per 2.0 g soil of soil samples in the presence of 0.1% (v/v) acetone Δ and 10 mg/liter CAT \circ (A) and 1.0 mg/liter Triton X-155 Δ and H_2O \circ (B) distributed according to time in hours. Substrates are added to equal $\text{WHC}_{60\%}$ and O_2 consumption rates are corrected for changes reflected by an empty flask \circ containing 1.0 ml H_2O (C).

Dox agar slant. Similarly, soil from a chlordane containing flask (Flask No. 2) was also inoculated into the enrichment agar. Growth occurred on both slants and this was subsequently streaked on 100 mg/liter CAT-Czapek Dox agar plates so as to obtain isolated colonies. Again, the resulting colonies were small, hard, and glistening white in appearance and random examination of several colonies revealed Gram negative bacilli occurring singly and in small chains. Several of these colonies were then subcultured onto 100 mg/liter CAT-Czapek Dox agar slants and Bacto-Stock Culture agar slants with the organisms isolated from Flasks 2 and 3 receiving the temporary designation of C-1 and A-1, respectively. Fresh subcultures of A-1 and C-1 were set monthly thereafter as described previously.

The confirming respirometer study of organism C-1 revealed that the bacillus was capable of utilizing some component(s) of the chlordane-acetone-Triton X-155 carbon source (Figure 3). It would appear that acetone was utilized more effeciently than the chlordane-acetone-Triton X-155 substrate (Figure 3).

Organisms A-1 and C-1 were incapable of growth on Czapek Dox agar without a carbon source, 10 mg/liter Triton X-155 Czapek Dox agar, and exhibited light growth on 1.0% acetone Czapek Dox agar. Good growth was obtained



Figures 3A and B. Oxygen consumption in $\mu\text{l O}_2/\text{hr}$ per 2.2 ml culture of cultures in the presence of 1.0% (\bar{v}/v) acetone Δ and 100 mg/liter CAT O (A) distributed according to time in hours. Corrected for changes reflected by an empty flask O containing 1.0 ml H_2O (B).

on 100 mg/liter CS-Czapek Dox agar. Thus, it appeared that the organism in question was capable of degrading and utilizing chlordane in some manner. It is of interest that acetone was utilized and significance of this will be documented in the "Discussion".

Organisms A-1 and C-1 were subjected to the extensive tests listed in "Materials and Methods" and they were found to be identical in all respects. However, in the final stages of the characterization, blood agar plates were streaked with organisms A-1 and C-1 and the resulting growth was found to be composed of two distinctively different colonial morphological types. This procedure was repeated to rule out the possibility of contamination and the existence of two morphologically different colonies was confirmed. Thus, "organisms" A-1 and C-1 were actually mixed cultures of two organisms; both organisms present in A-1 and in C-1. It is theorized that separation of organisms was effected in the original isolation. However, in selecting several of what appeared to be identical colonies, stock cultures of two similar organisms were obtained. The fact that A-1 and C-1 were mixed cultures explains two difficulties encountered in the preliminary phases of this study: (1) the unreproducibility of biochemical tests performed on A-1 and C-1 which are not described in the study, and (2) the shape of

the two respirometer growth curves (Figures 2 and 3) and the pH curve (Figure 4). The effect of the mixed culture on these growth curves will be discussed in the "Discussion".

The blood agar isolants differed both in their colonial morphology and in their hemolytic capabilities. One organism formed very small (approximately 1 mm in diameter) white colonies on the blood agar and exhibited no hemolysis. This organism was designated as W-C1 or W-A1 depending on whether it was isolated from the original C-1 or A-1. However, since subsequent tests confirmed that both W-C1 and W-A1 were identical in every respect, these organisms will be considered as one organism and called "Organism W". The other organism formed slightly larger colonies on the blood agar and was beta hemolytic. The colonies were bicolor in appearance, having a white irregular periphery and a dark green center. This organism was also present in cultures A-1 and C-1 and will be designated as "Organism BC", since biochemical tests revealed no differences between isolants BC-A1 and BC-C1.

Organism W was found to be a Gram negative, polar flagellated bacillus measuring 0.5x1.6-2.8 microns. The bacillus occurred singly, occasionally in pairs, but seldom in chains of three organisms. Capsules were demonstrated with both the capsular stain and the Gram

stain techniques as viewed with a conventional light microscope. The capsules were also present in the hanging drop preparation as observed with the phase contrast microscope. The organism exhibited a moderate degree of motility in the hanging drop preparations. In the Bacto-SIM medium, motility was limited to the upper 5 mm of the medium suggesting strict aerobic requirements. When present in liquid media, such as Bacto-Tryptic Soy broth and carbohydrate broths, W formed a moderately thick cream colored, mucoid pellicle approaching 4.0 mm in thickness if undisturbed. Upon swirling, the pellicle would settle, but it would not break apart, and persisted in the bottom of the tube. If undisturbed, the pellicle would eventually settle in the bottom. Teasing of the pellicle with inoculating needles resulted in breaking it up into mucoid, slimy strings. Phase contrast microscopic examination of a thin pellicle revealed no organized gross or fine structure and showed it to be merely a closely packed mass of cells. Cells at the periphery of the pellicle would occasionally break loose and swim into the surrounding liquid of the hanging drop preparation. Depending upon the liquid media used, growth in the early stages, prior to pellicle formation, would be exhibited as uniform turbidity or flocculant particles throughout the broth. Pellicle formation was

never observed in chlordane broth.

Organism W was capable of growing in Bacto-Tryptic Soy broth over a pH range of 4.0 to 9.5 and tolerated NaCl concentrations up to 3.5%. It was noted that, in Bacto-Tryptic Soy broth having concentrations of 1.0-2.5%, pellicle formation was enhanced. Upon swirling, the pellicle persisted as an intact, dry-looking, membranous sheet. However, teasing with inoculating needles revealed the true mucoid nature of the pellicle formed by W.

After three successive transfers, W still exhibited growth at 3 C and 40 C with no growth being demonstrable below 3 C or above 40 C.

Colonial morphology of Organism W on Bacto-Blood Agar Base enriched with 5.0% defibrinated human blood, Bacto-Tryptic Soy agar, and 100 mg/liter CS-CFBAM with agar present as 1.0% was similar except for colony size. The colonies formed by W on these agar media after 48 hours incubation at 28 C were small (1.0 mm in diameter), white, round, entire, convex, smooth, and glistening. The colonies were difficult to remove from the surface of the agar media due to their mucoid consistency. No pigment formation was noted on any of these media and, on 100 mg/liter CS-CFBAM, the colonies were less than 1.0 mm in diameter and were very hard.

Organism W produced a yellow-green pigment on King's chromogenic agar B which fluoresced brilliantly under ultraviolet light. The pigment appeared to be water soluble as it permeated the entire agar slant. The same fluorescent pigment was noted for W on Bacto-Tryptic Soy-4% instant nonfat milk agar plates. No pigment production was demonstrable for W on King's chromogenic agar A. Organism W also produced the fluorescent pigment on Bacto-Tryptic Soy agar enriched with 10% egg white if the inoculum was from a subculture possessing the pigment. However, this pigment was not demonstrable on any other medium and, when the egg white agar was inoculated with growth from Bacto-Stock Culture Agar slants, the pigment production could not be elicited. Thus, it appeared that the ability to produce the pigment was easily lost.¹

Organism W produced acid from various carbohydrates but, typically, a weeks incubation was required before the acid production could be regarded as significant. Ethanol and propanol were apparently oxidized by this organism since there was no acid formed within the Durham vials. Utilization of glucose, lactose, and sucrose was also oxidative, since no growth occurred in

¹King, Ward, and Raney, loc. cit. Garibaldi, loc. cit.

the petrolatum sealed tubes prepared after Hugh and Lelfson¹ (Table 2). These carbohydrate tests, along with the other biochemical tests (Tables 3 and 4) and morphological characteristics, tentatively place Organism W in the genus Pseudomonas and henceforth, this organism will be referred to as Pseudomonas W. It could not be positively identified with any species listed in Bergey's Manual of Determinative Bacteriology.²

Organism BC was also characterized as a Gram negative bacillus. It was polar flagellated and measured 0.4-0.5x2.2-2.9 microns. This organism occurred as single bacilli with occasional pairs being demonstrable. Capsule formation was also noted for BC on capsular stain and Gram stain preparations viewed with a conventional light microscope. Phase contrast examination of a hanging drop preparation also revealed the presence of capsules. BC was very actively motile in hanging drop preparations and also seemed to be strongly aerobic as determined by its confined motility in Bacto-SIM medium. Organism BC also formed a pellicle in complex liquid media but not in chlordanes broths. The pellicle formed by BC was also cream colored but it was thicker than those formed by

¹Hugh and Lelfson, loc. cit.

²Robert S. Breed (ed.), Bergey's Manual of Determinative Bacteriology (seventh edition; Baltimore: The Williams and Wilkins Company, 1957) pp. 35-212.

TABLE II
CARBOHYDRATE UTILIZATION

Carbohydrate ¹	<u>Pseudomonas</u> W	<u>Pseudomonas</u> BC
Acetone	Alk ²	NC ³
Ethanol	A ⁴	Alk
Fructose	A	Alk
Galactose	A	Alk
Glucose	A	Alk
Glucose Fermentative	NG ⁵	NG
Glucose Oxidative	A	Alk-A ⁶
Glycerol	NC	Alk
Lactose	Alk	Alk
Lactose Fermentative	NG	NG
Lactose Oxidative	A	Alk
Maltose	Alk	Alk
Mannitol	Alk	Alk
n-Propanol	A	Alk
Starch	Alk	Alk
Sucrose	Alk	Alk
Sucrose Fermentative	NG	NG
Sucrose Oxidative	A	NC
Xylose	A	NC

¹Carbohydrates were present as 1.0% concentrations in Bacto-Phenol Red Broth Base and dispensed in Durham fermentation tubes with the exception of the oxidative and fermentative glucose, lactose, and sucrose which were prepared according to Hugh and Leifson (see appendix).

²Alk=alkaline ³NC=no change

⁴A=acid ⁵NG=no growth

⁶Alk-A=Initially alkaline changing to acid

TABLE III
AMINO ACID UTILIZATION¹

Amino Acid	<u>Pseudomonas W</u>	<u>Pseudomonas BC</u>
DL - Alanine	+ ²	- ³
L+ - Arginine	+	-
DL - Asparagine	+	+
DL - Aspartic	-	-
Cysteine	+	+
L - Glutamic	+	+
Glycine	+	-
DL - Leucine	+	+
DL - Lysine	+	-
DL - Methionine	+	-
DL - Phenylalanine	+	+
DL - Proline	+	+
DL - Serine	+	+
DL - Threonine	+	-
DL - Tryptophan	+	-
DL - Tyrosine	-	-
DL - Valine	+	+

¹ Amino acids present as 0.01M amino acid in the
Cl free basal medium

² +=growth

³ -=no growth

TABLE IV
MISCELLANEOUS BIOCHEMICAL TESTS

Test	<u>Pseudomonas</u> W	<u>Pseudomonas</u> BC
Indole	-	-
Methyl Red	-	-
Voges Proskauer	-	-
Citrate	+	+
H ₂ S (SIM)	-	-
H ₂ S (Kligler's)	-	-
Nitrate Reduction	-	+
Casein Hydrolysis	+	+
Litmus Milk	Alk ⁴ , Red ⁵ , Pep ⁶	Alk, Red, Pep
Geletin Liquefaction	-	+
Urea Hydrolysis	-	-
Starch Hydrolysis (agar)	-	-
Phenylalanine Deamination*	-	-
Arginine Deamination ¹ ,*	+	-
Acetamide Deamination*	-	+
Cytochrome Oxidase I ² ,*	+	-
Cytochrome Oxidase II ³ ,*	+	-
Catalase Production	+	+
Hemolysis	None	Beta
0.1% Casamino Acids*	+	+
Temperature Preference	25-30 C	25-30 C
Temperature Tolerance	3-40 C	10-42 C
pH Tolerance	4.0-9.5	3.5-11.0
Salinity Tolerance	3.5%	6.0%

¹Anaerobic deamination ²Kovac's Oxidase Test

³Gaby and Hadley's Oxidase Test as modified by
Ewing and Johnson

⁴Alk=alkaline

⁵Red=reduction of litmus

⁶Pep=peptonization

*=see appendix

Organism W in comparable media of identical incubation, temperature, and times. The BC pellicle was capable of reaching thicknesses of up to 8.0 mm and, upon swirling, the pellicle would break up into small "globs" and strings of a mucoid-like material. This pellicle also settled to the bottom of culture tubes with time and, when teased with inoculating needles, it could be pulled into long strands of slimy, mucoid material. Phase contrast examination of a hanging drop preparation of a thin BC pellicle also revealed no organized gross or fine structure. As with the W pellicle, cells at the periphery of the pellicle were capable of breaking loose and a high degree of motility was noted. Early growth in liquid media was also manifested as either uniform turbidity or as mucoid particles floating in the media.

Organism BC exhibited more resistance to pH extremes in Bacto-Tryptic Soy broth than did W and growth was detected at pH 3.5-11.0. Salt tolerance was much more pronounced with BC exhibiting detectable growth in Bacto-Tryptic Soy broth having a NaCl concentration of 6.0%. As with Organism W, high salt content seemed to elicit a marked increase in pellicle formation. Pellicles formed by BC measured up to 1.0 cm in thickness and, upon swirling, the pellicle could not be easily disrupted. Vigorous swirling resulted in long, mucoid

ropes being thrown out from the main pellicle mass. In no case was the pellicle formed in broths having greater than normal salt concentration broken up into fragments.

Organism BC was capable of growth, after three successive transfers at the stated temperatures, at 10 and 42 C. No detectable growth occurred at temperatures outside of these limits.

The organism formed small (1.0 mm in diameter), cream colored, convex, round, entire, smooth, and glistening colonies of mucoid consistency on Bacto-Tryptic Soy agar. The morphology of the colonies on Bacto-Blood Agar Base enriched with 5.0% defibrinated human blood was similar except that the periphery of the colonies was slightly undulated and white in color while the center of the colony exhibited a gray-green color. Extensive beta hemolysis was also observed as was not the case with Organism W on the blood agar. Colonial morphology of Organism BC on 100 mg/liter CS-CFBAM containing 1.0% agar was identical to that of Organism W.

Organism BC did not exhibit pigment production on any agar medium with one exception. There was a brown pigment produced on King's chromogenic agar B after a period of approximately five weeks. The agar slants had been incubated at 28 C for two weeks, at the end of which time, there was no detectable pigment. The agar slants

were then placed in the refrigerator for storage. Three weeks later, the tubes were removed, and, a brown pigment had formed on the surface of the slants and the growth was a dark brown. The brown pigment did not appear to be water soluble as it did not diffuse into the agar.¹

Organism BC was completely refractory towards carbohydrates with growth in the carbohydrate media effecting, in most cases, a pronounced alkalinity (Table 2). Unlike Pseudomonas W, Organism BC was capable of reducing nitrate to nitrite and exhibited stratiform geletin liquefaction. This organism is also probably best placed within the genus Pseudomonas based on data from morphological characteristics and biochemical tests (Tables 2, 3, and 4). However, the reactions of this organism also preclude positive identification with any known species recognized in Bergey's Manual.²

The pathogenicity of the two pseudomonads for white mice was determined by inoculating, intraperitoneally, two mice each with W, BC, and sterile physiological saline (W and BC were washed twice and suspended in sterile physiological saline, the two suspensions being adjusted to equal optical densities). Neither Pseudomonas

¹King, Ward, and Raney, loc. cit. Garibaldi, loc. cit.

²Breed, loc. cit.

W nor Pseudomonas BC was pathogenic for white mice as evidenced by no death and no erythema or lesions at the site of inoculation.

The two pseudomonads were subjected to a test to determine whether or not they could utilize acetone, chlordane, Triton X-155, and agar as sole carbon sources. These media were prepared as described in "Materials and Methods" with the exception that CFBAM was substituted for the Czapek Dox basal agar medium. This change was necessitated by the fact that the available nitrogen in Czapek Dox is present as nitrate and Pseudomonas W cannot reduce nitrates. Both Pseudomonas W and Pseudomonas BC exhibited heavy growth on 100 mg/liter CS-CFBAM with little or no growth occurring on 1.0% acetone CFBAM. The growth of W and BC could not be elicited on similar agar media containing Triton X-155 or agar only as the sole source of carbon. Since the presence of acetone did increase the oxygen consumption of soil samples and cultures (Figures 2A and 3A), and the methyl ketone test showed that a negligible amount of acetone was present in the agar medium, another method was chosen to detect acetone utilization. A 10% (v/v) aqueous acetone solution was prepared by adding unsterile acetone to sterile water. A sufficient amount of this solution to yield a 1.0% (v/v) acetone broth was then added to sterile CFBBM

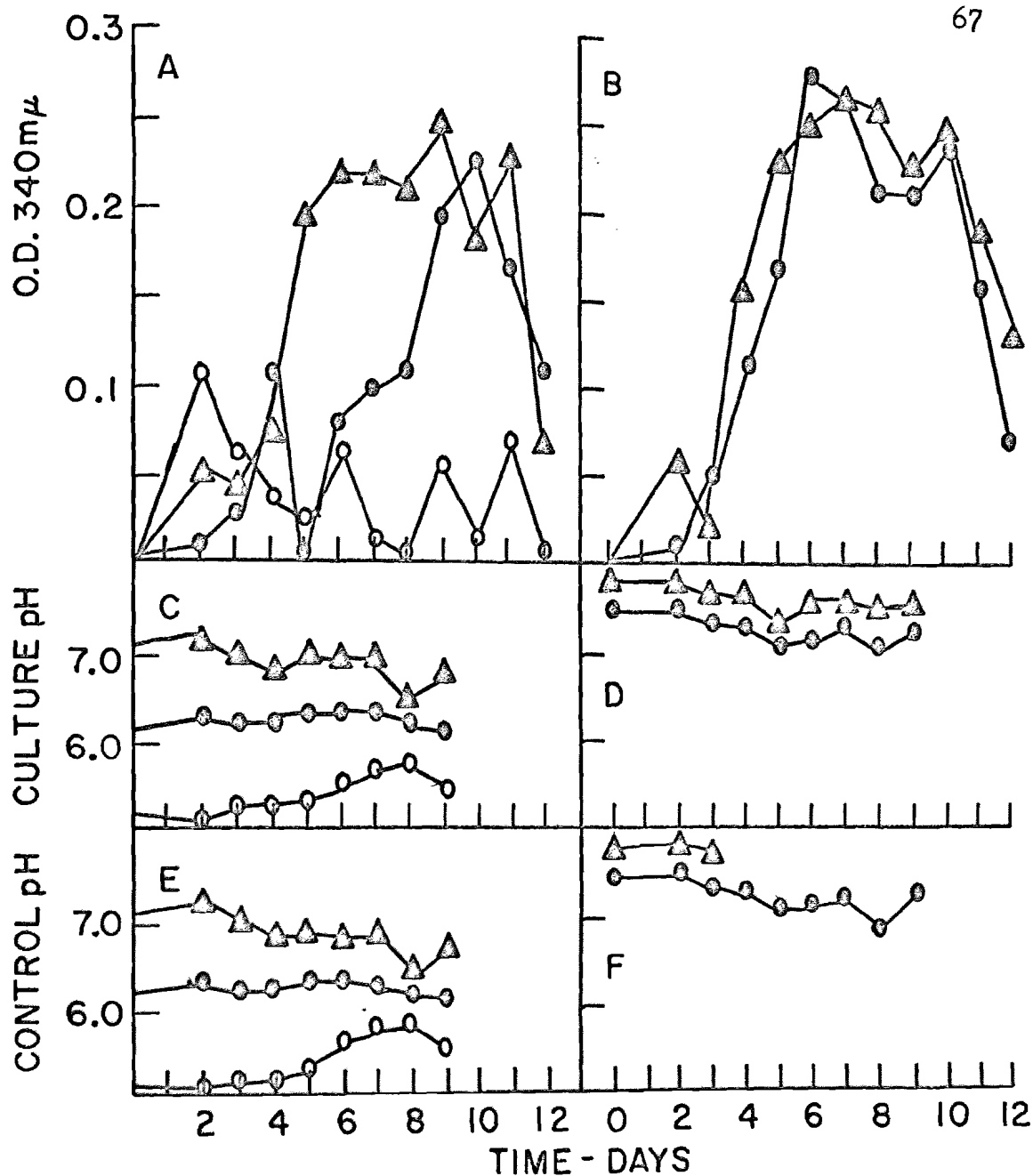
present in screw cap tubes. The sterility of the acetone broth was then checked by incubation at 28 C for 36 hours and the sterile acetone broth was then inoculated with organisms W and BC. Throughout the entire procedure, the caps were kept tightly screwed down to prevent loss of the highly volatile acetone. The caps were loosened daily so as to prevent the development of an oxygen tension below the optimum for the aerobic bacilli. Both pseudomonads, W and BC, were capable of utilizing acetone as a sole source of carbon and energy as demonstrated by this method.

Throughout the course of the study, it was observed that the best growth occurred within the temperature limits of 25-30 C. This was true for growth on all types of chlordane media as well as for growth on complex media. Therefore, 28 C was chosen as the optimum temperature for chlordane degradation.

Growth in 100 mg/liter CAT-Czapek Dox broth as a function of hydrogen ion concentration was determined before it was known that "Organism C-1" was actually a mixed culture of the two pseudomonads. Time prevented repeating this phase of the study using pure cultures as the inoculum. However, the results are presented here and are considered significant. Justification for presenting this data will be described in the "Discussion"

of this study. The optimum pH was found to be the medium which was measured as pH 7.90 prior to inoculation (Figure 4B). This pH afforded the most rapid logarithmic growth phase and, once the maximum stationary phase was obtained, it persisted for some time. The medium buffered at the initial pH of 7.50 exhibited a greater optical density at its maximum stationary phase than did that of the pH 7.90 medium (Figure 4B). However, this phase was not attained as rapidly in the pH 7.90 medium, nor did it persist as long once it was reached (Figure 4B). The buffering capacity at each pH was found to be effective since, although pH varied from day to day, the relative pH values of the inoculated media (Figures 4C and D) versus uninoculated media (Figures 4E and F) remained constant. In addition to the turbidometric characterization of growth, Gram stains made on the inoculated flasks confirmed the presence of Gram negative bacilli at the end of four days incubation. Further evidence of growth was obtained when a loop of culture from each flask was introduced into Bacto-Tryptic Soy Broth. The presence of organisms in all of the inoculated pH media was verified by growth occurring in the complex broth. This procedure also revealed the contamination of one uninoculated control flask.

Pseudomonas W, Pseudomonas BC, and mixtures of the



Figures 4A, B, C, D, E, and F. Turbidometric characterization (optical density at 340 mμ) of growth in 100 mg/liter CAT as a function of pH 5.15 ○, 6.15 ◐, 7.10 Δ (A), 7.50 ○, and 7.90 Δ (B) distributed according to time in days. Changes in pH distributed according to time in days expressed for inoculated media of pH 5.15 ○, 6.15 ◐, 7.10 Δ (C), 7.50 ○, and 7.90 Δ (D) and corresponding uninoculated controls (E and F).

two organisms were capable of utilizing 0.0001 mg/liter through 1000 mg/liter CAT-CFBBM. No growth occurred at 10,000 mg/liter chlordane and the lower limit on growth as defined by chlordane concentration was not determined. Turbidometric quantitation of the growth with time proved unsuccessful for two reasons: (1) the organisms formed curd-like flocs in the broth which did not contribute to turbidity, and (2) the 1000 and 100 mg/liter broths decreased in turbidity as compared to the uninoculated controls. When the 1000 and 100 mg/liter chlordane controls were set to read 0.3 optical density (O. D.) at 400 millimicrons by adjusting the 100% light transmission control on the Spectronic 20, inoculated broths exhibited lower O. D.'s (greater per cent light transmittancies). The O. D.'s obtained for cultures of Pseudomonas W in 1000 and 100 mg/liter CAT-CFBBM were 0.20 and 0.06, respectively. Pseudomonas BC, in the 1000 and 100 mg/liter chlordane broths, exhibited O. D.'s of 0.00 and 0.15, respectively. Mixtures of the two pseudomonads also clarified the broths and the O. D.'s obtained for the 1000 and 100 mg/liter broths were 0.09 and 0.05, respectively. The stated O. D.'s were mean values of duplicate runs and were obtained at the end of two weeks incubation. Thus, in utilizing the chlordane, the turbidity of the broths was reduced, in some way, by

the pseudomonads. However, this phenomenon was not observed with CAT agar plates, or at least the clarification of CAT agar was not detectable. It should also be noted that an aqueous solution of 10 mg/liter Triton X-155 and 1.0% (v/v) acetone was clear, and opacity resulted only after the addition of chlordane.

CHAPTER V

DISCUSSION

The two Pseudomonas species, W and BC, were capable of utilizing technical chlordane as their sole source of carbon and energy. This conclusion was based on the ability of chlordane-silica agar (100 mg/liter CS-CFBAM) to support growth of the organisms in pure culture. Pseudomonas BC evidently was capable of degrading and utilizing the chlordane in the Czapek Dox basal salts medium which had nitrate as the only available nitrogen source. It is theorized that, prior to the point when A-1 and C-1 were found to be identical mixed cultures each composed of W and BC, Pseudomonas W remained viable on the CS-Czapek Dox agar slant subcultures only because it was provided with required ammonium ions by the nitrate reducing enzymes of Pseudomonas BC. This theory perhaps explains, in part, the shape of the growth curves obtained in the two respirometer studies and the pH study (Figures 2, 3, and 4). Examination of Figures 2A and B indicate that some factor has produced two, fairly distinct, logarithmic growth phases, as evidenced by the chlordane, acetone, and Triton X-155 flasks. The presence of two logarithmic phases is even more pronounced, and significant, in Figure 3A as revealed by the oxygen consumption rate

exhibited by the acetone control flask. The presence of two distinctly different exponential phases in growth, as exhibited by the chlordane containing system graphed in Figure 3A, was less apparent and the significance of the peaks is questionable. Figure 4A also reveals the presence of two logarithmic phases in the growth curves of the pH 6.15, 7.50, and 7.90 flasks. Again, the presence of two exponential phases in the other flasks is questionable. It is proposed that two logarithmic growth phases were demonstrable in these studies for the following reasons:

1. Mixed cultures of Pseudomonas W and Pseudomonas BC were, unknowingly, used for the three studies.
2. With the exception of the screening respirometer study (Table 1 and Figure 2), the only nitrogen source present was nitrate.
3. Since Pseudomonas W was incapable of reducing nitrate, the first exponential phase was an expression of Pseudomonas BC replication. During this time nitrate was being reduced to ammonium ions.
4. The second exponential phase resulted from the presence of ammonium ions and their subsequent utilization by Pseudomonas W.

5. Since a dualistic exponential growth phase was exhibited by the first respirometer study (Figures 2A and B), and it is assumed that available ammonium ions would be present in the soil for utilization by Pseudomonas W, other factors may be involved. Such factors could include the presence of many complex food stuffs in the soil and growth of organisms not utilizing the exogenous, but rather endogenous, substrates. However, the dualistic logarithmic phases of Figures 2A and B are not pronounced and may not be significant as compared to the dualistic exponential phase exhibited in Figure 3A.
6. The presence of two exponential phases of growth in Figures 3A, 4A, and 4B was interpreted as significant. The probable explanation for a lack of two very distinct logarithmic growth phases in these figures, with the exception of the acetone containing flask in Figure 3A, is that more than one utilizable carbon source was present. This introduced two variables not present in the acetone flask of Figure 3A. Thus, it is possible that there was preferential utilization of the various carbon sources accounting for some of the peaks noticed in the

growth curves.

The inoculum used for the characterization of growth in 100 mg/liter CAT-Czapek Dox basal broth as a function of pH was a mixed culture of the two pseudomonads. This phase of the study was undertaken prior to the time that it was known organisms "A-1" and "C-1" were mixed cultures, and time prevented repeating this study using both pure and mixed cultures. However, it is felt that this study is not invalidated by the fact that a mixed culture was used. It is suspected that Pseudomonas W and Pseudomonas BC function in a symbiotic manner in the degradation of chlordanes. This suspicion was based on the observation that, in 100 mg/liter CAT-CFBBM and 100 mg/liter CAT-Czapek Dox basal broth, growth seemed to be heavier and more rapid when the inoculum was a mixed culture. There has been no attempt to verify this theory but present plans are to follow the growth of both pure and mixed cultures of the pseudomonads in 100 mg/liter CAT-CFBBM by the determination of cellular nitrogen. Thus, pH 7.50 to 8.00 was chosen as the optimum pH for chlordanes biodegradation.

It was noted that growth of both pseudomonads in the various CAT liquid media usually occurred as small (1-4 mm) brown-yellow curds in the bottom of flasks incubated in a shaker-water bath at 28 C. The presence of Gram negative bacilli was verified by the examination of

smears made from various curds. Although this unusual pattern of growth seems to contradict the strong aerobic requirements of both W and BC, the two phenomena can be reconciled. The encapsulation of both organisms accounted for the formation of the curd-like growth which would settle due to its mass. Agitation of the flasks saturated the relatively shallow liquid medium with oxygen allowing growth to occur in the depths of the medium. On the basis of curd formation, identification of the two pseudomonads with the genus Zoogloea has not been dismissed. Thus, a river water sample, heavily polluted with untreated domestic sewage, was inoculated with W and BC. Figure 5 shows, photographically, the results of this test, along with two 100 mg/liter CAT-CFBBM flasks inoculated with Pseudomonas W. The flocs formed by Pseudomonas BC were not as large as those of Pseudomonas W and a flask of 100 mg/liter CAT-CFBBM inoculated with BC was not included in Figure 5 due to the non-photogenic nature of the flocs.

It was mentioned previously that both organisms were capable of utilizing acetone and data is available to support this contention (Figure 3A and Table 3). Pursuit of this observation was beyond the scope of the study, however, this utilization is in agreement with studies performed by Leadbetter and Foster, in 1960. They found that the obligate methane utilizing bacterium

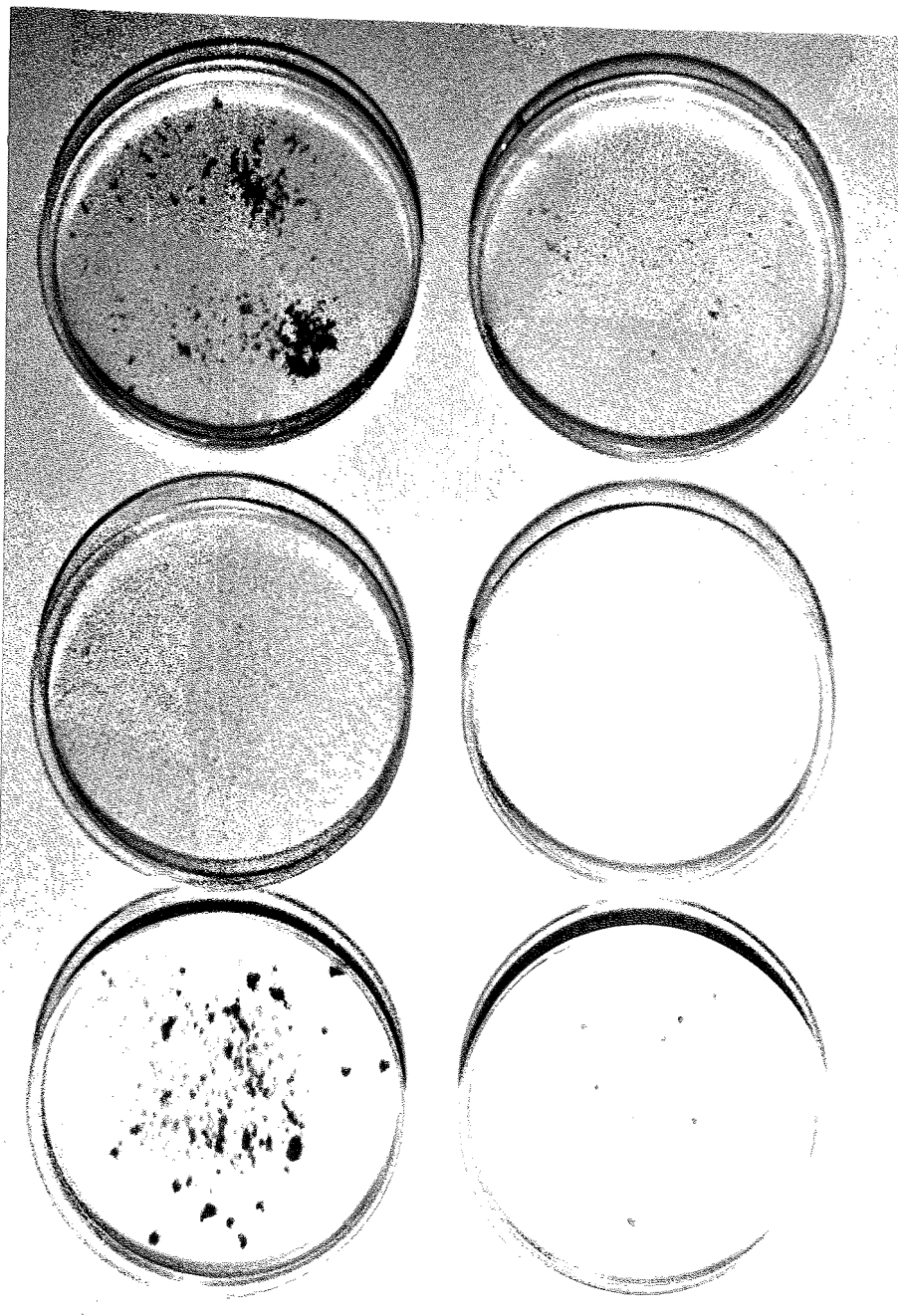


Figure 5. Photograph of the flocs formed in sterile river water polluted with untreated domestic sewage and inoculated with Pseudomonas BC (top left) and Pseudomonas W (bottom left) and of the flocs formed by Pseudomonas W in 100 mg/liter CAT-CFBBM (top and bottom right). Uninoculated controls of the river water (left middle) and the 100 mg/liter CAT-CFBBM (right middle) are shown.

Pseudomonas methanica, along with various unidentified Gram negative bacilli isolated from the soil, were capable of forming acetone from propane containing cultures. Propionic acid was also formed, but it was found not to be interconvertable with acetone.¹ A subsequent study by Foster, in 1962, demonstrated that Mycobacterium smegmatis 422, when grown on propane, could utilize propane, acetone, and n-propanol. Foster thus proposed the following metabolic pathway: propane to acetone to acetol.²

During pilot studies, various chlordane liquid media were examined for their ability to support the growth of organisms. It was noticed that, although some growth occurred in chlordane-silica liquid media as measured turbidometrically, the growth was very light as compared to growth in CAT media. This could be accounted for, in part, by the fact that additional utilizable carbon sources are present in the CAT media which enhance growth. However, another explanation is that adsorption of the chlordane to silica for in vitro cultivation effectively precludes enzymatic attack. This

¹E. R. Leadbetter and J. W. Foster, "Bacterial Oxidation of Gaseous Alkanes" Archiv fur Mikrobiologie, XXXV (January, 1960), 92-104.

²J. W. Foster, "Hydrocarbons as Substrates for Microorganisms," Antonie Van Leeuwenhoek Journal of Microbiology and Serology, XXVIII (1962), 241-274.

statement is based on the reasons proposed by Alexander for the recalcitrance of pesticides towards biodegradation.¹

The results of this study necessitate further investigations and they are briefly outlined as follows:

1. It has been observed that, when carried on complex media such as Bacto-Stock Culture agar, neither Pseudomonas is capable of growing in the various chlordane media, or else does so after a very prolonged lag growth phase. Thus, a study to determine the inducibility of the chlordane degrading enzyme system would prove valuable. Such a study could be implemented by measuring the oxygen consumption of cell free extracts of cells grown on chlordane-free, complex media. Adaptability of the chlordane degrading enzyme system to other chlorinated hydrocarbons could also be investigated at this point.
2. It is desirable to determine which component(s) of technical grade chlordane is actually being degraded and utilized. Such a study would require analytical grade chemicals of the various components of technical chlordane.

¹M. Alexander, "Biodegradation; Problems of Molecular Recalcitrance and Microbial Fallibility," Advances in Applied Microbiology, VII (1965), 35-77.

Growth could then be followed by determining total cell nitrogen while at the same time performing a quantitative analysis of chlordane (or the components of technical chlordane) concentration.¹ It would be expected that growth would be inversely proportional to chlordane concentration.

3. Following a study to determine which component(s) of technical chlordane is being degraded and utilized, it would next be advisable to identify the intermediate metabolites of the degradative pathway(s).
4. A study designed to demonstrate how the chloride ions are removed from the indan nucleus of the chlordane molecule would also be of great significance. An investigation of this nature would be difficult to implement, although a preliminary study to determine whether or not chloride ions appear in a chloride free medium would be the first step in such a study. This

¹T. G. Bowery, Chlordane, (Vol. II of Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives, ed. Gunter Zweig. New York: Academic Press, Inc., 1964), pp. 50-51. Bernard Davidow, "A Spectrophotometric Method for the Quantitative Estimation of Technical Chlordane," Journal of the Association of Official Agricultural Chemists, XXXIII (August, 1950), 886-894.

could be done by employing a quantitative analysis for the detection of chloride ions and, at the same time, growth as a function of cellular nitrogen could be followed. A positive correlation between free chloride ions and total cell nitrogen would be expected.¹

5. Perhaps the most important phase of future studies would involve extending the findings of this investigation, and those of the above proposals, to field conditions subject to the complexities of a natural environment.

¹H. L. Jensen, "Decomposition of Chloroacetates and Chloropropionates by Bacteria," Acta Agriculturae Scandinavica, X (1960), 83-103.

CHAPTER VI

SUMMARY

Chlorinated hydrocarbon insecticides are used extensively in the United States and their use is increasing. Many of these compounds are toxic to a wide variety of invertebrates and vertebrates and, at the same time, resist, or are recalcitrant to, chemical, physical, and biological degradation. A study was therefore undertaken to determine the biodegradability of the chlorinated hydrocarbon insecticide chlordane under laboratory conditions.

Two small, Gram negative, polar flagellated, encapsulated bacilli were isolated from the soil. They were both capable of utilizing chlordane as their sole source of carbon and energy. The optimum temperature and pH for the degradation appeared to fall within the ranges of 25-30 C and 7.5-8.0, respectively. Chlordane concentrations ranging from 0.0001 to 1000.0 mg/liter seemed to be utilized.

Tentative classification of the organisms places them within the genus Pseudomonas. The bacilli appear to be two distinct species types but neither can be identified with Pseudomonas species described in the literature.

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APPENDIX

APPENDIX

TECHNIQUES AND MEDIA FORMULATION

Adsorption of Technical Chlordane on to Colloidal Silica for the Preparation of Agar Media (Barvak, Alroy, and Mateles, loc. cit.)

1. 10.0 g technical chlordane is weighed out in a large beaker and then mixed with 50.0 ml ethyl ether.
2. The ether-chlordane solution is transferred to a large mortar containing 10.0 g colloidal silica and thoroughly mixed with the silica.
3. The ether is then allowed to evaporate at room temperature, leaving behind a fluffy, cream colored powder of one part chlordane to one part silica by weight.
4. An appropriate amount of this powder is then added to the basal salts agar medium to give the desired chlordane concentration.
5. The agar-chlordane-silica medium is then autoclaved and, prior to solidification in tubes or pouring into Petri dishes, the medium is agitated to insure uniform distribution of the silica.
6. The solidified agar medium is homogenous in appearance, almost translucent when present in thin layers (2-5 mm), and there was no detectable desorption of chlordane from the silica as a result of autoclaving.
7. This method is unsatisfactory for suspension of chlordane in liquid media. The silica settles with time unless agitated more vigorously than practical during incubation of inoculated media.

Oxidative-Fermentative Carbohydrate Medium (Hugh and Lelfson, loc. cit.)

Peptone	2.0 g
NaCl	5.0 g

K ₂ HPO ₄	0.3 g
Agar	3.0 g
Bromthymol Blue (1% aqueous solution)	3.0 ml
Distilled H ₂ O	1000.0 ml
Adjust pH to 7.1		

Dispense the above into tubes, autoclave at 15 psi for 15 minutes, and, after sterilization, add enough filter sterilized 10% aqueous carbohydrate solution to yield a 1.0% carbohydrate medium. Allow to solidify in an upright position.

Two tubes of the medium are stabbed with a young culture and one tube is layered (5-19 mm) with melted sterile petrolatum.

The formation of acid in the open tube indicates oxidative utilization of the carbohydrate. Acid formation in both tubes demonstrates fermentative utilization.

Cytochrome Oxidase Detection (Edwards and Ewing, loc. cit.)

Reagent A:

1% alpha naphthol in 95% ethanol

Reagent B:

1% aqueous para-aminodimethylaniline HCl
(this reagent should be prepared weekly and refrigerated)

Two or three drops of reagents A and B are allowed to flow over the growth on agar slants or colonies on plates. The presence of cytochrome oxidase is indicated by the growth or colonies turning blue within two minutes. Color formation after two minutes is regarded as negative.

Cytochrome Oxidase Detection (Kovacs, loc. cit.)

Reagent:

1% aqueous tetramethyl-p-phenylenediamine HCl

A few drops of the test reagent are added to the surface of agar growth. Positive cultures develop a pink color which becomes successively dark red, purple, and finally black in 10 to 30 minutes.

Medium for the detection of anaerobic arginine utilization
by Pseudomonas (Thornley, loc. cit.)

Bacto-Peptide	1.0 g
NaCl	5.0 g
K ₂ HPO ₄	0.3 g
Agar	3.0 g
Phenol Red (1% aqueous solution)	1.0 ml
L(+)-Arginine HCl	10.0 g
Distilled H ₂ O	1000.0 ml

Dispense the above into tubes, autoclave at 15 psi for 15 minutes, and allow to solidify in the upright position.

Two tubes of the medium are stabbed with a young culture and one tube is layered (5-10 mm) with melted sterile petrolatum.

A strong alkaline reaction in the sealed tube appearing within 2-3 days is regarded as positive.

Medium for the detection of acetamide deamination
(Buhlmann, Vischer, and Bruhin, loc. cit.)

Bacto-Peptide	1.0 g
NaCl	5.0 g
KH ₂ PO ₄	2.0 g
Phenol Red (1% aqueous solution)	1.2 ml
Acetamide	10.0 g
Distilled H ₂ O	1000.0 ml
Adjust pH to 6.8 or 6.9	

Acetamide deamination is indicated by the medium turning from its original yellow color to a pink or purple within 4 to 5 days.

Medium for the detection of phenylalanine deamination
(Ewing, Davis, and Reavis, loc. cit.)

Yeast Extract	3.0 g
DL-phenylalanine	2.0 g
(or L-phenylalanine)	(1.0 g)
Na ₂ HPO ₄	1.0 g
NaCl	5.0 g

Agar 12.0 g
 Distilled H₂O 1000.0 ml

Dispense in tubes, autoclave at 15 psi for 10 minutes,
 and allow to solidify in a slanted position.

Inoculate and incubate for 24 hours. Allow 4 or
 5 drops of 10% aqueous ferric chloride to run
 down over the growth on the slant. If phenyl-
 pyruvate has been formed from phenylalanine a
 green color develops in the syneresis fluid and agar.

Media for the enhancement of the pigments pyocyanin and
 fluorescein (King, Ward, and Raney, loc. cit.)

Medium A

Bacto-Peptone 20.0 g
 Glycerol, C. P. 10.0 ml
 MgCl₂ (anhydrous) 1.4 g
 K₂SO₄ (anhydrous) 10.0 g
 Bacto-Agar 15.0 g
 Distilled H₂O 1000.0 ml
 Adjust pH to 7.2

Medium B

Proteose peptone No. 3, Difco 20.0 g
 Glycerol, C. P. 10.0 ml
 K₂HPO₄ 1.5 g
 MgSO₄·7H₂O 1.5 g
 Bacto-Agar 15.0 g
 Distilled H₂O 1000.0 ml
 Adjust pH to 7.2

Dispense A and B in tubes, autoclave at 15 psi for
 15 minutes, and allow to solidify in the slanted
 position.

Inoculate and incubate at the organisms temperature
 preference. Recommended incubation schedule is as
 follows:

Medium A - 48 hours or longer at 37 C
 Medium B - 24 hours at 37 C followed by 2 to 3
 days at room temperature. (Pigment formation
 may be temperature dependent)

Medium A enhances pyocyanin production and Medium B
 that of fluorescein.

Medium for the enhancement of fluorescent pigment production by Pseudomonas species (Garibaldi, loc. cit.)

The amount of Bacto-Tryptic Soy agar required to prepare 100.0 ml is added to 90.0 ml of H₂O and autoclaved at 15 psi for 15 minutes. (Any commercial complex agar media will work equally as well.)

An egg is prepared by scrubbing the surface lightly with soap and water and then rinsing in water. The egg is then dipped in 95% ethanol, and, after 5 minutes, the egg is removed, allowed to drain, and the remaining alcohol is removed by flaming the shell.

A small surface area of the egg shell is painted with tincture of merthiolate and this area of the shell is removed with sterile forceps. Ten milliliters of egg white are then removed with a sterile 10 ml pipette having a tip bore large enough to allow easy passage of the viscous egg white.

The 10 ml of egg white is added to the sterile Bacto-Tryptic Soy agar prior to solidification. (If a more homogenous agar is desired, the egg white may be blended in a sterile blender prior to addition to the agar.) In either case, the melted agar must be cool enough to handle comfortably with bare hands (approximately 45 C) before the egg white is added. The 10% egg white Bacto-Tryptic Soy agar is then mixed thoroughly and poured into plates. Plates should be incubated for at least 24 hours before using, to insure sterility.

According to Garibaldi, the action of this agar medium is based on the ability of the egg white to chelate iron as fluorescent pigment production has been shown to be inversely proportional to iron concentration.